

MITOCHONDRIAL UPTAKE OF ANTHOCYANIDINS AND PROTECTION FROM OXIDATIVE STRESS

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Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
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ABSTRACT

The anthocyanins show efficient antioxidant properties and free radical scavenging properties which result in various health-promoting benefits. This research investigated the ability of anthocyanidins to distribute into mitochondria and protect mitochondria from oxidative stress.

In an *in vitro* study, the uptake of pure cyanidin and quercetin, and their 3-glucosylated forms into isolated rat liver mitochondria was tested, along with their effects on mitochondrial oxidative stress parameters. The absorption of cyanidin was significantly higher (67% uptake of 125 μ M) than the other three flavonoids. Measurements indicated that the cyanidin was taken up into or tightly bound by mitochondria. Also, results suggested that cyanidin uptake was partially dependent on membrane potential. When incubated together (internally and externally) with mitochondria all tested flavonoids decreased reactive oxygen species (ROS) generation during mitochondrial respiration, and inhibited lipid peroxidation to different extents. Importantly, pre-loaded CY showed much stronger effects against oxidative stress in two analyses than other flavonoids. Due to its greater uptake by mitochondria, cyanidin may provide greater protection *in vivo*.

In an *in vivo* study, cyanidin, quercetin and their 3-glucosides were administered into rat tail vein to give a dose of 7.6 μ mol/Kg body weight. Cyanidin and its glucoside had greater affinity to liver and kidney than did quercetin and its glucoside; particularly, all test tissues contained a significantly higher amount of cyanidin than

other test flavonoids. Also, cyanidin accumulated more in liver mitochondria than other flavonoids, and consistent with *in vitro* results was present in mitochondria to a much greater extent than cyanidin glucoside. However, delivery of the flavonoids at this dose did not significantly affect the liver mitochondria susceptibility to lipid peroxidation or the level of endogenous tissue oxidative damage.

Altogether the results show that cyanidin can rapidly and efficiently accumulate in mitochondria, wherein it exhibits strong bio-antioxidant activity against oxidative stress and may help protect mitochondrial function and integrity. Also, the anthocyanidin and its 3-glucoside have greater ability than flavonols to accumulate in organs; especially cyanidin presented in liver mitochondria to a much greater extent. Cyanidin could be a potent natural antioxidant compound that is effective in mitochondria-protective therapies.

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LIST OF ABBREVIATIONS

•OH	hydroxyl radical
DP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
C3G	cyanidin 3-glucoside
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CHD	coronary heart disease
CY	cyanidin
DAD	diode array detector
DCFDA	2',7'-dichlorofluorescein diacetate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Fe ²⁺	ferrous ion
Fe ³⁺	ferric ion
H ₂ O ₂	hydrogen peroxide
HCl	hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid
HO ₂ •	hydroperoxyl radical
HOCl	hypochlorous acid
HPLC	high performance liquid chromatography
L•	lipid carbon-centered radical
LDL	low density lipoprotein
LH	polyunsaturated fatty acid
LO•	lipid alkoxyl radical
LOH	lipid alcohol
LOO•	lipid peroxyl radical
LOOH	lipid hydroperoxide
MDA	malondialdehyde
MgCl ₂	magnesium chloride
MTP	mitochondrial permeability transition
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotine adenine dinucleotide phosphate(reduced form)
NO	nitric oxide
O ₂ ^{•-}	superoxide anion
ONOO ⁻	peroxynitrite

PBS	phosphate buffered saline
Q3G	quercetin 3-glucoside
QU	quercetin
RLM	rat liver mitochondria
RNA	ribonucleic acid
RNS	reactive nitrogen species
RO [•]	alkoxy radical
ROO [•]	peroxyl radical
ROOH	alkyl hydroperoxide
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
<i>t</i> -buOOH	<i>tert</i> -butyl-hydroperoxide
Triton X-100	C ₁₄ H ₂₂ O(C ₂ H ₄ O) _{<i>n</i>}
UV	ultraviolet

1. INTRODUCTION

1.1 Mitochondria in health and disease

There is substantial evidence that mitochondria-derived reactive oxygen species (ROS) cause oxidative damage to biological macromolecules and contribute to aging and a range of human diseases including cancer, diabetes, obesity, stroke, and neurodegenerative diseases (Armstrong, 2008; Davidson, 2010; de Moura et al., 2010; Harman, 1972; Ladiges et al., 2010; Witte et al., 2010). The mitochondrial respiratory chain is considered as a major source of ROS, which increase with age (Bandy & Davison, 1999; Ladiges et al., 2010; Witte et al., 2010).

Excess production of ROS results in significant oxidative stress in the mitochondria, can trigger mitochondrial dysfunction and cell death, and is implicated in ageing and many diseases (Pieczenik & Neustadt, 2007). It has been demonstrated that ROS can increase oxidative damage to lipid and proteins; also, cause damage and high mutation rates of mitochondrial DNA (mtDNA) and alterations of the expression of several clusters of genes in ageing tissues and senescent cells (Ladiges et al., 2010). Preventing mitochondrial damage and reducing the release of active oxygen species could efficiently limit age-related oxidative stress and disease. Therefore, antioxidants which can directly target and have effect in the mitochondria will most efficiently prevent or decrease the oxidative damage (Smith et al., 2008).

1.2 Protection against oxidative stress and disease by flavonoids and anthocyanins

Flavonoids have gained considerable attention recently, particularly because of their antioxidant and radical-scavenging activities. It has been documented that

flavonoids have efficiency against oxidative damage and related diseases (Darvesh et al., 2010; Havsteen, 2002). Particularly, anthocyanin-rich red and purple berries have attracted considerable interest and extensive mechanistic studies for their potential protective effects and health benefits (Andres-Lacueva et al., 2005; Hassimotto et al., 2008; Neto, 2007; Serraino et al., 2003). The anthocyanins are unique among the flavonoids in not being negatively charged at physiological pH, and show efficient antioxidant and free radical scavenging properties (Kong et al., 2003). The anthocyanins have an array of health-promoting benefits as they can protect against a variety of oxidants through several mechanisms.

Studies have shown that anthocyanins have some beneficial health effects such as reducing age-associated oxidative stress, possessing anti-inflammatory properties, preventing cancer, and protecting against vascular diseases (Dai et al., 2009; Kong et al., 2003; Neto, 2007; Thomasset et al., 2009b). Also, anthocyanins have several peripheral effects including: stabilizing connective tissue, promoting collagen formation and helping to prevent oxidative damage to blood vessels (Andres-Lacueva et al., 2005; Chrubasik et al., 2010).

The antioxidant activity of anthocyanins and their other functions has been well studied *in vitro*, as well as the correlation between their antioxidant capacity and chemical structure (Kong et al., 2003). However, there are still fewer studies as compared to studies of other flavonoids. The antioxidant efficacy *in vivo* of anthocyanins has also been less thoroughly documented, possibly due to the limited sources of pure anthocyanins and the associated cost. Moreover, information on whether they could enter cells and accumulate in subcellular organelles such as

mitochondria in the specific tissues and organs and its effects becomes a major question. Results from these types of studies will contribute to our understanding of the potential significance of anthocyanins in human health.

Interest in the *in vivo* bioavailability of anthocyanins arises from evidence supporting a role for these flavonoids in human health. Anthocyanins have an affinity for, and accumulate in tissues such as kidney and skin (Matsumoto et al., 2006a; Matsumoto et al., 2006b). For further discussion on *in vivo* functions of anthocyanins, it is critical to know the physiological uptake and distribution of each anthocyanin in tissues and intracellular deposition perhaps especially in mitochondria.

Studies on the anthocyanin absorption and metabolism in tissues were reported elsewhere in both human and experimental animals (Bub et al., 2001; Kalt et al., 2008; Matsumoto et al., 2001; Mazza et al., 2002; Miyazawa et al., 1999; Talavéra et al., 2005). In these studies significant anthocyanin contents were found in ocular, brain, kidney and liver tissue, as well as plasma and urine, after either oral or intravenous treatment mainly using berry fruits extract as the anthocyanin source (Andres-Lacueva et al., 2005; Ichiyanagi et al., 2006; Matsumoto et al., 2006b; Talavéra et al., 2005).

However, there are no data on the potential ability of anthocyanins to reach and be taken up in the mitochondria. As well, such studies using fruit extracts as anthocyanin sources are not suitable for precise discussion of anthocyanin metabolism. I thus studied the uptake of anthocyanins in isolated mitochondria and distribution in experimental animals using pure anthocyanins.

2. LITERATURE REVIEW

2.1 Mitochondria in health and disease

Mitochondria, as the cell's powerhouse, occupy a unique position in controlling the life and death of a cell. Physiologically, mitochondria perform a variety of key cellular functions, including ATP production, intracellular Ca^{2+} regulation, reactive oxygen species (ROS) generation, and apoptosis (DiMauro, 2004; Hoyer et al., 2008; Reddy, 2006; Seddon et al., 2007; Zeviani et al., 1989). Importantly, as the mitochondria play a dominant role in processing oxygen and then converting substances from foods into ATP that powers most cell functions, if problems happen within the mitochondria, it could result in many health problems. Mitochondrial damage and /or dysfunction disrupt the function of cells, tissues, and organs and contribute to a remarkably wide range of diseases (DiMauro, 2004).

The mitochondrial respiratory chain (complexes I-V) provides the oxidative metabolic pathway in the cell, and its biosynthesis is controlled by the mitochondrial genome (mtDNA) and the nuclear genome (nDNA) (DiMauro, 2010). Mutations of mitochondrial DNA damage mitochondrial function and lead to a number of mitochondrial related diseases. For instance, mitochondrial or nuclear DNA mutation can influence the energy production ability of the mitochondria, and consequently causes certain types of disease such as MELAS (Mitochondrial Encephalomyopathy) and MERRF (Myoclonic Epilepsy with Ragged Red Fibers) (Armstrong, 2007; DiMauro, 2004). In these diseases, the mitochondrial defect usually affects the muscles, cerebrum, or nerves because these cells require the most energy.

As well as diseases directly caused by mitochondrial DNA mutation, mitochondrial oxidative damage contributes to the pathology of many other disorders, such as cardiovascular and neurodegenerative disease (Armstrong, 2007; DiMauro, 2004, 2010; Newsholme et al., 2007).

Although the mitochondrial free radical theory of ageing has recently come into question, characteristic decreases in mitochondrial function and increases in oxidative stress with age are well documented and accepted (Hekimi et al., 2011; Lapointe & Hekimi, 2010; Salmon et al., 2010). In the ageing process a number of changes in mitochondria occur, such as respiratory function decline along with a concomitant increase in production of reactive oxygen species; in particular, mtDNA mutations and oxidative damage are increased in ageing human tissues (Lee & Wei, 2007; Muller-Hocker, 1992; Wei, 1998; Wei et al., 2001).

2.2 The concept of oxidative stress and mitochondrial disease

2.2.1 Reactive oxygen species (ROS)

Free radicals derived from oxygen represent the most important group of radical species generated in living systems (Miller et al., 1990). Normally, oxygen free radicals or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are formed during a variety of biochemical reactions and cellular functions (Valko et al., 2007). Physiological concentrations are required for normal cell functions including intracellular signaling and redox regulation (Nordberg & Arner, 2001). However, excessive quantities of ROS can be very harmful to living systems. Overproduction of ROS causes oxidative damage, which leads to loss of cell function and apoptosis or necrosis (Zamzami et al., 1997). Also, accumulated ROS generation

contributes to the physiology of ageing (Halliwell & Gutteridge, 1999; Valko et al., 2007; Valko et al., 2006) .

Several reactive oxygen species are formed in the human body and include: the superoxide radical ($O_2^{\bullet-}$); hydrogen peroxide (H_2O_2); hypochlorous acid ($HOCl$); the hydroxyl radical ($\bullet OH$); peroxy ion (O_2^{2-}); hydroperoxyl radical ($HO_2\bullet$); lipid peroxy radicals ($LOO\bullet$); and lipid hydroperoxide ($LOOH$) (Groff & Gropper, 2000). ROS can readily react with most biomolecules, and start a chain reaction of free radical formation. In order to stop this chain reaction and further consequent damage, the formed radical must either react with another free radical, eliminating the unpaired electrons, or react with a free radical scavenger— a chain-breaking or primary antioxidant (Halliwell, 1999; Nordberg & Arner, 2001).

2.2.2 Oxidative stress contributes to the development and pathology of many human diseases

Oxidative stress is a state where excess ROS overpower endogenous antioxidant systems and cause damage to all components of the cell, including proteins, lipids, and DNA. Such damage triggers cell dysfunction and death; and consequently it applies in various disease states and ageing (Valko et al., 2007).

Oxidative stress has been associated with numerous human diseases including Alzheimer's, amyotrophic lateral sclerosis (ALS), Parkinson's, atherosclerosis, ischemia/reperfusion neuronal injuries, degenerative disease of the human temporomandibular joint, cataract formation, macular degeneration, degenerative retinal damage, rheumatoid arthritis, multiple sclerosis, muscular dystrophy, and cancers (Christen, 2000; Davi et al., 2005; Hitchon & El-Gabalawy, 2004; Milam et al., 1998; Nunomura et al., 2006; Rando et al., 1998; Varma et al., 1995; Wood-Kaczmar et

al., 2006). A good example is how oxidative stress has an effect in cardiovascular disease. Studies document that oxidation of low density lipoprotein (LDL) initially drives the atherogenesis process, which leads to atherosclerosis formation, and finally results in cardiovascular diseases (Aviram, 2000; Van Gaal et al., 2006). In addition, lipid peroxidation occurs at the biological tissues even in healthy organisms, and contributes to ageing and oxidative stress related disease (Halliwell & Chirico, 1993; Negre-Salvayre et al., 2010).

2.2.3 Oxidative damage in mitochondria

2.2.3.1 Mitochondria are a major source of ROS generation

ROS are generated within the cell and within mitochondria through endogenous enzymatic and non-enzymatic reactions (Finkel & Holbrook, 2000). Mitochondrial sources include the electron transport chain which passes electrons from the electron carriers through the respiratory chain to oxygen, and monoamine oxidase (MAO) at the outer membrane. Cytosolic sources of ROS are xanthine oxidase (XO) and cytochrome P450 reductases. Plasma membrane-linked sources of ROS include the NADPH oxidases and cytochrome P450 (Armstrong, 2008; Halliwell, 2006). ROS can also arise outside the cell, including singlet oxygen ($^1\text{O}_2$) generation by UV irradiation, environmental ozone (O_3) or other ROS and RNS in pollutants, such as cigarette smoke (Victor & Rocha, 2007).

The most important source of ROS under normal conditions in aerobic organisms is the leakage of activated oxygen from mitochondria, which carry out oxidation reactions during normal oxidative respiration (Halliwell, 2001). Indeed, much experimental evidence strongly suggests that leakage of electrons from the electron transport chain,

located in the inner mitochondrial membrane, is a major source of ROS in living cells ((Bandy & Davison, 1990; Peterside et al., 2003). The details of ROS species generation in mitochondria are well discussed in several papers (Cadenas & Davies, 2000; DiMauro, 2004; Halliwell, 2006; Halliwell & Gutteridge, 1999; Murphy, 2009; Murphy & Smith, 2007; Valko et al., 2007).

Superoxide anion ($O_2^{\bullet-}$), considered the “primary” ROS, is generated mostly within the mitochondrial complexes I and III (Cadenas & Sies, 1998; Valko et al., 2007). $O_2^{\bullet-}$ can be subsequently converted to other reactive species, including hydrogen peroxide (H_2O_2), which can further react to form the highly reactive hydroxyl radical ($\bullet OH$) via the Fenton reaction (Raha & Robinson, 2000; Valko et al., 2007). Hydroxyl radical has strong reactivity with biomolecules, and is considered more dangerous to biological systems than any other ROS (Nordberg & Arner, 2001). Importantly, the strong oxidants including peroxy radicals ($ROO\bullet$) derived from these toxic electron transport chain reactions initiate fatty acid peroxidation.

2.2.3.2 Mitochondria are susceptible to oxidative damage

Mitochondria are considered the most important source of ROS production in mammalian organs under normal conditions (Cadenas & Davies, 2000; Kowaltowski & Vercesi, 1999; Skulachev, 1996), making mitochondria especially vulnerable to oxidative damage. Mitochondrial components are exposed to a relatively high amount of ROS. Evidence indicates that, *in vitro*, mitochondria convert 1–2% of the oxygen molecules consumed into superoxide anions at intermediate steps of the mitochondrial respiratory chain (Kowaltowski & Vercesi, 1999; Skulachev, 1996). Also, it has been shown that the steady state concentration of $O_2^{\bullet-}$ in the mitochondrial matrix is about 5-

to 10-fold higher than that in the cytosolic and nuclear spaces (Cadenas & Davies, 2000).

ROS can result in significant damage to mitochondrial components and initiate degradative processes. Consequences of this damage include modification to proteins, lipids and DNA, which thereby disrupt mitochondrial function and also cause more ROS to be released to the cytosol (Murphy & Smith, 2007; Pieczenik & Neustadt, 2007).

2.2.3.3 Oxidative damage to mitochondrial DNA, protein and lipid.

Mitochondrial DNA (mtDNA) is a more sensitive target for oxygen radical attack than nuclear DNA (nDNA) (Cadenas & Davies, 2000; Richter et al., 1988). mtDNA damage can lead to dysfunction of the mitochondrial respiratory chain, which further stimulates the overproduction of ROS and additional mtDNA damage ((Bandy & Davison, 1990), and consequently apoptosis may occur (Ozawa, 1997). Indeed, mtDNA fragmentation is associated with ageing (Muller-Hocker, 1992; Ozawa, 1998).

Mitochondrial ROS interact directly with a wide variety of mitochondrial proteins, and cause mitochondrial protein regulatory and pathological dysfunction (Page et al., 2010). Mitochondrial enzyme proteins are essentially all susceptible to attack by ROS (Kowaltowski & Vercesi, 1999; Raha & Robinson, 2000). Studies have demonstrated that $O_2^{\bullet-}$, H_2O_2 and $\bullet OH$ inhibit enzyme activities in different regions of the electron transport chain. NADH dehydrogenase, succinate dehydrogenase, and ATPase activities are rapidly damaged by the above mentioned ROS (Newsholme et al., 2007; Zhang et al., 1990).

In addition, in the presence of Ca^{2+} , the oxidation of membrane protein thiols causes inner membrane permeabilization, which is referred to as the mitochondrial permeability transition (MPT) (Kowaltowski & Vercesi, 1999). MPT involves MPT pores opening, and leads to more ROS generation, and mitochondrial swelling, which triggers cell death in the intrinsic pathway of apoptosis (Fiskum, 2000). MPT allows Ca^{2+} to leave the mitochondrion, which causes further stress in the cell (Brookes et al., 2004).

Lipid peroxidation results in major oxidative damage to biological membranes (Halliwell & Chirico, 1993). Oxidation of fatty acids can cause membrane alterations (even in healthy organisms), increased membrane fluidity and permeability, efflux of cytosolic solutes and loss of membrane protein activities under oxidative stress (Avery, 2011). Mitochondrial generated ROS not only could attack DNA and protein, but also polyunsaturated fatty acids present in the mitochondrial membrane.

Mitochondrial membrane lipid peroxidation significantly damage mitochondrial respiration, oxidative phosphorylation and ion transport functions (Kowaltowski & Vercesi, 1999; Pieczenik & Neustadt, 2007). Importantly, lipid peroxidation damage complex I and induces MTP (Murphy & Smith, 2007). Also, ROS can degrade polyunsaturated lipids to form the highly reactive compound malondialdehyde (MDA), which reacts with the DNA building blocks, deoxyguanosine and deoxyadenosine so as to form DNA adducts, which contribute significantly to the risk of cancer (Davi et al., 2005; Kowaltowski & Vercesi, 1999; Marnett, 1999).

Moreover, oxidative damage could increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome *c* to the cytosol, and thereby activate cell apoptosis (Zamzami et al., 1997).

2.2.3.4 Oxidative damage cause mitochondrial diseases

Significantly, mitochondrial mutations and dysfunction caused by oxidative damage trigger necrosis and apoptosis of cells and are implicated in a number of diseases ((Bandy & Davison, 1990; DiMauro, 2004; Zamzami et al., 1997). For example, liver failure is often associated with mitochondrial DNA (mtDNA) depletion syndromes (Dubern et al., 2001). It is a vicious cycle among oxidative stress and mitochondrial dysfunction. As oxidative stress occurs, it causes mitochondrial DNA mutations, protein modifications, enzymatic abnormalities and lipid peroxidation, which in turn fuels oxidative damage; consequently resulting in mitochondrial dysfunction and cell death. There is remarkable realization that impairments of mitochondria caused by acute and chronic oxidative stress are the key component of mitochondria disease etiology (Sheu et al., 2006).

Indeed, it has been suggested that generation of oxidants by mitochondria, and damage of mitochondrial components and functions, are important aspects of the ageing process (Ashok & Ali, 1999) . In nearly all diseases caused by mitochondrial dysfunction, ROS metabolism is one of the crucial factors (DiMauro, 2004, 2010).As mitochondrial damage leads to significant health risk, a therapy to decrease oxidative damage would be useful in a range of clinical situations. Prevention of mitochondrial damage and reduction in the release of ROS could efficiently limit mitochondria-related diseases and ageing. Antioxidants which can target and have effect in the

mitochondria will most efficiently prevent or decrease the oxidative damage in mitochondria; a continued search for better and more effective antioxidants to prevent or decrease mitochondrial damage is needed.

2.3 Antioxidants against oxidative stress

The complex interplay between free radicals and intracellular antioxidant systems is very important for cell functions and living systems. As oxidative stress is considered to be an important part of many diseases, many types of antioxidants are available for use in foods and in dietary supplements for the prevention of disease or as pharmacological treatments.

2.3.1 Physiological defense mechanisms and antioxidants

Antioxidant enzymes and low molecular weight antioxidants are two components that compose the antioxidant defense system in cells. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), thioredoxin and glutathione peroxidase (GPx); and the low molecular weight antioxidants include vitamins A and E, ascorbic acid (vitamin C), and glutathione (GSH) as well as many natural compounds such as polyphenols (Juurlink & Paterson, 1998; Nordberg & Arner, 2001; Valko et al., 2007). These substances are the body's natural defense against endogenously generated ROS and other free radicals. Their protective abilities against oxidative stress have been reported in numerous reviews and original papers (Avery, 2011; Fridovich, 1995; Halliwell, 1999, 2006; Valko et al., 2007). The human diet also contains many compounds of antioxidant nature, such as polyphenols; especially flavonoids, which have gained considerable attention, particularly because of their antioxidant and

radical-scavenging activities (Heim et al., 2002; Krishnaswamy & Raghuramulu, 1998).

2.3.2 Theory of mitochondria targeting

Given the mitochondrion's essential role in a wide range of human disease, a need exists to choose efficient antioxidants that protect mitochondrial functions. However, the administration of antioxidants has failed to show consistent and significant benefits in mitochondrially related diseases (Halliwell, 2011; Sheu et al., 2006). For example, vitamin E administration has showed no benefits in the treatment of Parkinson's diseases (The Parkinson Study Group, 1993, 1998).

The most reasonable explanation for antioxidants inefficacy is their limited bioavailability and/or distribution into the mitochondria (Fink et al., 2009; Halliwell, 2001; James et al., 2007; Sheu et al., 2006). Increasing the antioxidant capacity of the mitochondrial is a potential therapy against oxidative damage (Smith et al., 2003).

Nowadays, targeted antioxidants have been developed by conjugating the lipophilic triphenylphosphonium (TPP) cation (Figure 2.1) to an antioxidant moiety, such as the mitochondria-targeted derivative of ubiquinone (MitoQ) or the mitochondria-targeted derivative of α -tocopherol (MitoVit E) (Milagros Rocha & Victor, 2007; Sheu et al., 2006). These compounds pass easily through all biological membranes, accumulate in the cell, and further into the mitochondria due to the plasmalemmal and mitochondrial membrane potentials (Sheu et al., 2006; Smith et al., 1999). These compounds have yielded remarkable experimental results in mitochondria studies, and cells and tissues undergoing oxidative stress and apoptotic death (Dhanasekaran et al., 2004; Smith et al., 2008; Victor & Rocha, 2007).

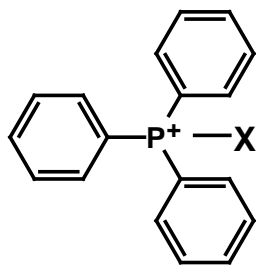


Figure 2.1 TPP+ cation X is a conjugated antioxidant moiety.

In order to exhibit mitochondrial targeting, the two major structural features of compounds are: a sufficient hydrophobic surface; and a positive charge. These two features allow direct permeation of the mitochondrial membranes so that substantial concentrations of the selected compounds within the mitochondria can occur (Ross et al., 2005).

In principle, any antioxidant with a lipophilic cation structure could be used for targeted delivery to the mitochondria (Armstrong, 2008). Anthocyanins would fit this model as they have similar structural features with the TPP cation in that they are aromatic, somewhat hydrophobic, and possess a positive charge (at specific pH conditions). Based on these structural features and their good antioxidant properties, I investigated both mitochondrial uptake and potential protection against oxidative stress of a select group of flavonoids.

2.4 Flavonoids

2.4.1 Overview

The aim of this section is to demonstrate that flavonoids; and specifically anthocyanidins, should be of great practical use against mitochondrial oxidative stress.

Flavonoids are a class of secondary plant polyphenolics with significant antioxidant and metal-chelating properties (Potter & Steinmetz, 1996). Flavonoids cannot be

synthesized by humans or other animals (Obrenovich et al., 2010). They are most concentrated in fruits, vegetables, nuts, spices, herbs, seeds, wines, teas and cocoa, and are consumed regularly in the human diet (Heim et al., 2002). With more developed analyses, flavonoids consumption for several countries had been estimated to total range from about 20 mg/day to 70 mg/day more recently (Beecher, 2003).

Numerous studies have strongly suggested that flavonoids exhibit health benefits including antioxidant, anticarcinogenic, antimutagenic, antiallergic, and anti-inflammatory properties, and pharmacological effects such as antituberculosis, antimalarial, antimicrobial and antiviral (Darvesh et al., 2010; Havsteen, 2002; Hu et al., 2006; Middleton et al., 2000). Indeed, a flavonoid rich diet has been linked with lower risk of heart disease and cancer (Knoops et al., 2004). The attention on the possible health benefits of flavonoids has risen quickly, mostly because of their potent antioxidant abilities and free-radical scavenging activities (Obrenovich et al., 2010)

Epidemiological studies provide insight that flavonoid-rich foods are associated with decreased chronic diseases such as cardiovascular disease, cancer, stroke, and even may have an effect on ageing (Bazzano et al., 2003; Dauchet & Dallongeville, 2008; Eichholzer et al., 2001; Mink et al., 2007).

A decreased risk coronary heart disease (CHD) mortality was strongly and inversely associated with flavonol and flavone intake in the Zutphen Elderly Study where more than a 50% reduction in mortality risk was found (Hertog et al., 1993). A 10-year follow-up of the same group found that a high flavonoid intake was correlated with a lower incidence of coronary disease (Hertog et al., 1997). Similar findings were shown

in a cohort study of 5,133 Finnish men and women where increasing flavonoid intake reduced CHD mortality (Knekt et al., 1996).

Additionally, flavonoid intake showed a noticeable reduction in the risk of cancer and type 2 diabetes (Zunino, 2009). A case-control study from Spain showed that total flavonoid intake was inversely associated with gastric cancer (Garcia-Closas et al., 1999).

2.4.2 Structure/Subclasses

Over 6400 natural flavonoids have been characterized from various plants. Flavonoids are classified according to their chemical structure, and are usually subdivided into the subgroups shown in Table 2.1. The basic structure of a flavonoid (Figure 2.2) is comprised of two benzene rings (A and B), which are linked through a heterocyclic pyran or pyrone (with a double bond) ring (C).

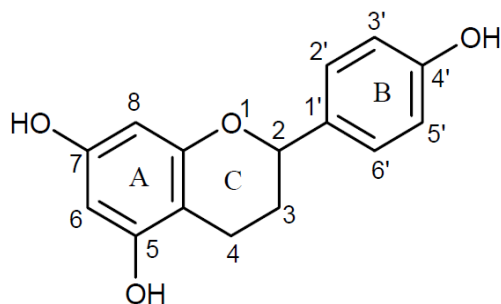


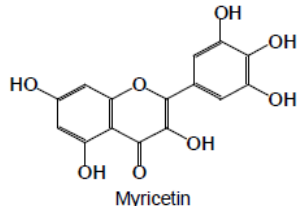
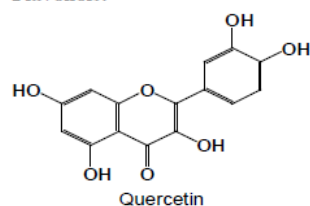
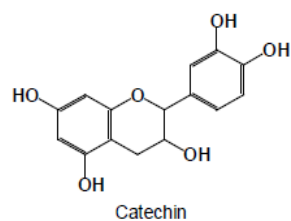
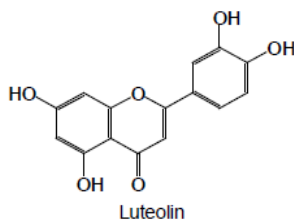
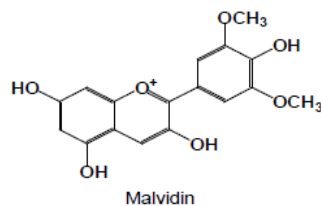
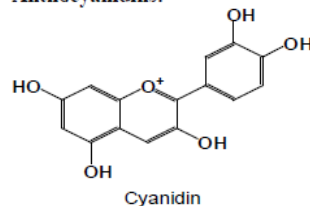
Figure 2.2 Basic flavonoid structure.

Flavonoids can be further subdivided into eight major subclasses based upon variations in the heterocyclic C-ring (Beecher, 2003). There are six major subclasses (Table 2.1) including flavones, flavonols, flavanones, catechins, anthocyanidins, and isoflavones; as well as two minor ones, the dihydroflavonols and chalcones (Heim et al., 2002; Peluso, 2006).

Table 2.1 Major flavonoid subclasses, chemical characteristics, and typical food sources. (Beecher, 2003)

Flavonoid subclass	B-ring position to C-ring	C-ring unsaturation	C-ring functional groups	Food flavonoids	Major food sources
Flavanol	2	None	3-hydroxy	Catechins Epicatechins Epigallocatechin	Teas, red grapes and red wines
Flavanones	2	None	4-oxo	Eriodictyol Hesperetin Naringenin	Citrus foods
Flavones	2	2-3 double bond	4-oxo	Apigenin Luteolin Tangeritin	Green leafy spices
Isoflavones	3	2-3 double bond	4-oxo	Biochanin A Coumestrol Daidzein Daidzin Genistein	Soy beans, soy food, legumes
Flavonols	2	2-3 double bond	3-hydroxy, 4-oxo	Kaempferol Myricetin Pachypodol Quercetin	Apple, onions, berries, citrus fruits
Anthocyanidins	2	1-2, 3-4 double bounds	3-hydroxy	Cyanidin Delphinidin Malvidin Pelargonidin	Red, purple and blue grapes and berries

Most dietary flavonoids occur in food as water-soluble O-glycosides, in which carbohydrate moieties attach to one or more hydroxyl groups; and with smaller amounts of more hydrophobic aglycones (Peluso, 2006). Attached carbohydrate moieties include D-glucose, L-rhamnose, D-glucorhamnose, D-galactose, lignin, and D-arabinose (Heim et al., 2002).

Flavonols:**Flavanols:****Flavones:****Isoflavones:****Anthocyanidins:****Figure 2.3 Structures of some common flavonoids.****2.4.3 Absorption and bioavailability**

The nutritional and or/medicinal potential of flavonoids can be affected by limited systemic and target tissue bioavailability and rapid metabolism (Obrenovich et al., 2010). For a better understanding of the important effects of flavonoids in human health and disease, a number of literature reviews of flavonoids metabolism and disposition are available (Griffiths, 1982; Hackett, 1986; Hollman & Katan, 1998).

The degree of absorption of individual flavonoid subclasses are different, and depend on several factors such as, chemical structure and location of absorption (Heim et al., 2002; Tapiero et al., 2002). It has been established that flavonoids enter the gastrointestinal tract, and absorption processes occur in the small intestine and colon (Scalbert & Williamson, 2000). Generally, flavonoid glycosides have to be

enzymatically deglycosylated prior to absorption and further metabolized by the liver; whereas aglycones are absorbed directly from the small intestine and then secreted in bile and urine (Griffiths, 1982; Hollman & Katan, 1997). However, some evidence suggests that anthocyanin glycosides are absorbed intact (Cao & Prior, 1999; Matsumoto et al., 2001).

Also, the presence of carbohydrate residues affects the physicochemical properties of the molecule and thus their ability to cross membranes and enter cells (Hollman & Katan, 1999). In addition, isoflavones have the highest bioavailability followed by flavanols, flavanones and flavonol glycosides (Passamonti et al., 2009).

Animal and human trials provide sufficient evidence that flavonoids are rapidly absorbed in the body and distributed in the tissues (Ali et al., 2005; de Boer et al., 2005; Kalt et al., 2008; Matsumoto et al., 2006a; Rein et al., 2000; Reinboth et al., 2010). Also in human studies, experiments showed the relative increased concentrations of anthocyanins present in plasma, serum and urine after administration (Cao & Prior, 1999; Matsumoto et al., 2001; Mazza et al., 2002; McGhie et al., 2003). Still, the exact mechanism of flavonoid absorption and metabolism are unclear. Some types of flavonoids have received very limited study and detailed evidence is needed.

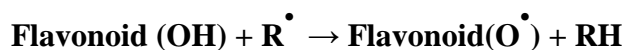
2.4.4. Protection against oxidative stress and disease

The research on flavonoid health benefits, particularly as it relates to oxidative stress has begun more recently as compared to other antioxidants. This section focuses on the flavonoid antioxidant ability and related potential benefit in prevention of oxidative stress and disease.

2.4.4.1 Antioxidant properties against oxidative damage

Flavonoids help protect against oxidative damage by contributing to the total antioxidant defense system along with antioxidant vitamins and enzymes. The antioxidant activity of flavonoids is regarded to be related to 1) scavenging free radicals, 2) chelating transition-metals involved in free-radical production and 3) inhibiting the enzymes participating in free-radical generation (Aruoma, 2003).

As shown below, flavonoids are able to donate a hydrogen atom from an aromatic hydroxyl group to a free radical, and scavenge reactive oxygen species by forming a more stabilized phenolic radical (Rice-Evans et al., 1996):



Where R^{\bullet} is an organic free radical and O^{\bullet} denotes an oxygen-centered radical (in the case of flavonoids, delocalized around the phenol ring).

Both the position of hydroxyl groups on the aromatic ring and their structural arrangement have an effect on their antioxidant and free radical scavenging activities (Peluso, 2006; Rice-Evans et al., 1996). An *o*-dihydroxy structure in the B ring offers higher stability to the radical form and participates in electron delocalization (Rice-Evans et al., 1996). The C2-C3 double bond, 3-OH group, and 4-oxo function in ring C of flavonoids exert the greatest radical scavenging potential (Rice-Evans et al., 1996).

Additionally, the number of hydroxyl groups is positively related to antioxidant activity; whereas substitution of hydroxyl groups by glycosylation decreases antioxidant activity (Havsteen, 2002; Rice-Evans et al., 1996). Quercetin, luteolin, myricetin, and kaempferol exhibited greater antioxidant capacity than glyco-conjugated flavonoids such as quercetin-3-glucoside, quercitrin, and rutin (Ioku et al., 1995; Noroozi et al., 1998).

Flavonoids have been shown to act as scavengers of various oxidizing species including hydroxyl, lipid alkoxyl, peroxy and superoxide radicals, and hypochlorous acid (Heim et al., 2002; Rice-Evans et al., 1996). Flavonoids also protect molecules against damage caused by peroxynitrite *in vitro* (Heijnen et al., 2001). The capacity of scavenging superoxide and peroxynitrite could capably protect endothelial dysfunction under acute oxidative stress (Akhlaghi & Bandy, 2009). By scavenging such reactive species, flavonoids suppress the oxidative reactions in the system and prevent further formation of reactive radicals.

Many flavonoids chelate transition metal ions such as iron and copper, decreasing their ability to promote reactive species formation, and thereby diminish the generated free radicals in the medium (El Hajji et al., 2006; Soczynska-Kordala et al., 2001). Quercetin and rutin were highly effective at complexing transition metal ions and inhibiting lipid membrane peroxidation.

Besides ROS scavenging, flavonoids inhibit the activity of enzymes participating in free-radical generation. Robinetin, rhamnetin, eupatorin and baicalein showed strong inhibition of NADH-oxidase activity (Hodnick et al., 1994). Studies indicated that dietary administration of anthocyanins, proanthocyanidins, or catechin oligomers prevented the increased expression of cardiac NADPH oxidase in rats fed with high-fructose diet (Al-Awwadi et al., 2005). Flavonoids also can inhibit xanthine oxidase and prevent formation of superoxide; flavones showed higher inhibitory activity than flavonols (Cos et al., 1998).

2.4.4.2 Interaction with cell membranes

Importantly, flavonoids could also exert the antioxidant properties through interacting with and penetrating cell membranes, where their action maintains membrane fluidity and integrity (El Hajji et al., 2006; Heim et al., 2002; Oteiza et al., 2005). Flavonoids can accumulate and position themselves at the lipid-water interface of membranes through hydrophilic and lipophilic interactions (Rice-Evans et al., 1996). When flavonoids interact with bilayers, those containing more hydrophobic moieties can position in the interior of the membrane, whereas those with more hydrophilic moieties can undergo hydrogen bond with the polar head groups of phospholipids and accumulate at the membrane interface (Oteiza et al., 2005). The result of these interactions can effectively diminish the access of ROS and disrupt the reaction of lipid radicals with other fatty acids, thus protecting the structure and function of membranes against lipid and protein oxidation (Oteiza et al., 2005; Rice-Evans et al., 1996).

Indeed, the hydrophilicity/hydrophobicity, the degree of flavanol oligomerization and the number of hydroxyl groups are related to the protective effects of flavonoids on membranes and lipid peroxidation (Heim et al., 2002).

2.4.4.3 Reinforcement of cellular antioxidants

The antioxidant abilities of flavonoids also apply in protection or enhancement of endogenous antioxidants (Elliott et al., 1992; Filipe et al., 2002; van Acker et al., 2000). Certain flavonoids (including quercetin, myricetin, and fisetin) were shown to decrease oxidative stress by inducing glutathione S-transferase (GST), an enzyme which is among those controlled by the antioxidant response element and which

protects cells against oxidative stress caused by hydrogen peroxide (Fiander & Schneider, 2000).

The flavonoids in biological systems also contribute to recycling and/or stabilizing other antioxidants. Flavonoids have the capacity to reduce the α -tocopheroxyl radical, regenerate α -tocopherol (Filipe et al., 2002; Rice-Evans et al., 1996). This action sufficiently maintains α -tocopherol levels for longer time periods, enhances the antioxidant capacity of the cell and biomembranes, and delays the initiation of lipid peroxidation since vitamin E scavenges primarily peroxy radicals and other reactive species such as alkoxy radicals.

Furthermore, the combination of flavonoids and ascorbate may cooperatively protect the membrane from lipid peroxidation in the mitochondria (Bandy & Bechara, 2001; Ratty & Das, 1988). Bandy and Bechara (2001) observed an ability of certain flavonoids to mediate electron transfer from ascorbate to a membrane interface. Conversely flavonoids can preserve ascorbic acid activity in a system by regenerating ascorbate from ascorbate radical (Cossins et al., 1998; Heim et al., 2002).

2.4.4.4 Potential medicinal benefits in oxidative stress

Both *in vivo* and *in vitro* investigations have confirmed the ability of flavonoids to help protect biosystems against free radicals and oxidative stress (Middleton et al., 2000). For example, after 2 hours of chocolate consumption, as a source of epicatechin and procyanidin; a significant increase in human plasma epicatechin concentrations was observed; with a significant increase (31%) in human plasma total antioxidant capacity ($p < 0.04$) and a decrease of 40% in plasma thiobarbituric acid reactive substances ($p < 0.01$) (Rein et al., 2000).

The effect of flavonoids on coronary artery diseases (CAD) arise from protection against low density lipoproteins (LDL). The flavonoids protect LDL against oxidation by reducing the generation of free radicals, inhibiting the generation of lipid hydroperoxides, sparing α -tocopherol, and chelating metal ions (de Whalley et al., 1990). Flavonoids protect LDL from oxidation thus reducing atherogenicity; which potentially reduces oxidative stress related diseases including vascular disorders and coronary artery disease (Middleton et al., 2000; Nijveldt et al., 2001). In addition, dietary flavonoids can inhibit platelet activation and aggregation (Williams & Clarkson, 1998). *In vivo* evidence indicates that the consumption of flavonoids significantly decreases parameters of human plasma oxidant status in a dose-dependent manner (Rein et al., 2000).

Flavonoids are also powerful chain-breaking antioxidants that protect cell membranes from oxidative damage (Oteiza et al., 2005). It was observed that flavonoids can replace α -tocopherol as a chain-breaking antioxidant in liver microsomal membranes (van Acker et al., 2000).

In addition to antioxidant properties, flavonoids have received considerable attention because of their antimutagenic, anticarcinogenic, antiallergic, and anti-inflammatory properties (Middleton & Kandaswami, 1992; Nijveldt et al., 2001). Overwhelming evidence suggests that flavonoids could reduce the incidence of certain cancers, cardiovascular and neurodegenerative diseases, DNA damage and even may have antiageing properties. Excellent reviews on their potential medicinal benefits have been published (Darvesh et al., 2010; Havsteen, 2002; Middleton et al., 2000; Obrenovich et al., 2010)

In short, the protective antioxidant properties of flavonoids in biological systems relate to their capacity to transfer electrons to free radicals, chelate metal catalysts, activate antioxidant enzymes, and enhance antioxidant capacity in the system by recycling and/or stabilizing antioxidant compounds. Protection by flavonoids against oxidative tissue damage has been determined by effects on lipid peroxidation, DNA fragmentation and protein oxidation. The capacity of flavonoids in these aspects provides strong evidence that flavonoids can apply in numerous disease conditions and have beneficial effects on human health.

2.5 Flavonoid effects on mitochondria

As we are interested in the antioxidant ability of flavonoids and their potential medical benefits, the question arises whether flavonoids would be effective in protecting mitochondria against oxidative damage and thus be a possible therapeutic application in mitochondrial diseases.

Numerous studies indicate the beneficial effects of flavonoids in biological systems, including subcellular systems such as mitochondria and microsomes (Cavallini et al., 1978; Haraguchi et al., 1996; Trumbeckaite et al., 2006). Fewer studies have documented their specific applications in mitochondrial functions. Several studies have reported *in vitro* protective effects of flavonoids against both enzymatic and nonenzymatic mitochondrial lipid peroxidation (Elingold et al., 2008; Middleton et al., 2000).

Experiments on isolated rat liver mitochondria indicated that flavonoids efficiently inhibited ADP/ Fe(II)-induced lipid peroxidation and were potent inhibitors of MPT in Ca^{2+} induced swelling, as well as of protein sulfhydryl oxidation (Santos et al.,

1998). In addition, the flavonoid isolated from the roots of *Dalea elegans* demonstrated significant antioxidant activity and inhibited NADPH-dependent enzymatic lipid peroxidation in rat liver mitochondria (Elingold et al., 2008). Several flavonoids also inhibited nonenzymatic lipid peroxidation induced by ascorbic acid and ferrous sulfate in mitochondria (Ratty & Das, 1988).

Also, flavonoids can inhibit key enzymes of mitochondrial respiration. Certain flavonoids show strong inhibition of NADH-oxidase activity, depending on their hydroxylation/ methoxylation structural features (Hodnick et al., 1994; Santos et al., 1998); Since NADH-oxidase is one of the main sites of ROS generation, this suggests that flavonoids could inhibit ROS generation in mitochondria, although it would also impair ATP production.

In membrane studies relevant to mitochondria, certain flavonoids mediated a protective effect of ascorbate on peroxide-bleaching of membrane-bound cytochrome *c*; also, quercetin combined with ascorbate could protect the membrane from cytochrome *c*-catalyzed lipid peroxidation (Bandy & Bechara, 2001). In other studies with this system from our laboratory (unpublished results), it was found that the anthocyanin cyanidin was especially effective in the cooperation with ascorbate.

Regarding mitochondrial therapeutics, flavonoid–TPP conjugates were developed and purposed to act as mitochondrial targeting antioxidants (Mattarei et al., 2008). Mitochondriotropic quercetin derivatives were synthesized by linking the 3-OH of quercetin to the TPP moiety cationic group (Mattarei et al., 2008). The experiment indicated those compounds can accumulate in mitochondria by a transmembrane potential-driven process. Rather than acting as antioxidants, they inhibited

mitochondrial ATPase activity, produced pro-oxidant activity and MPT, and induced apoptosis for fast-growing cells (Mattarei et al., 2008). As such they might be useful as anticancer agents, but further studies are needed of their bioactivities *in vivo*.

Since flavonoids are very effective antioxidants, they may have protective effects on mitochondrial mutation and dysfunction. For example, epigallocatechin-3-gallate (EGCG) and luteolin significantly restored the mitochondrial membrane potential and ATP levels, and lowered ROS production in amyloid- β induced mitochondrial dysfunction *in vitro* and *in vivo* (Dragicevic et al., 2011).

The biomedical properties of flavonoids have been intensely investigated in cellular functions; however, the uptake and effects of flavonoids in mitochondria is still unclear. The detailed study of their activities and utilization in mitochondria is thus a promising research topic, and more evidence of these compounds in mitochondrial targeting and potential benefits are necessary.

2.6 Anthocyanins

Anthocyanins and their aglycone derivatives (anthocyanidins) are an important group of flavonoids. Anthocyanins are pigments that are responsible for red, blue and violet colours in many plants. More recently, anthocyanins have attracted considerable interest and extensive mechanistic studies for their potential protective effects and health benefits.

2.6.1 Structural properties

The most abundant anthocyanins in plants are the glycosides of the six anthocyanidins: cyanidin, pelargonidin, peonidin, delphinidin, petunidin and malvidin (Kong et al., 2003). The most common glycosyl groups of anthocyanidins are glucose,

galactose and arabinose; glycosylation provides stability to the anthocyanins and often occurs at C-3, and C-5 (Welch et al., 2008). The hydroxylation and sugar substitution influence the anthocyanin color and properties.

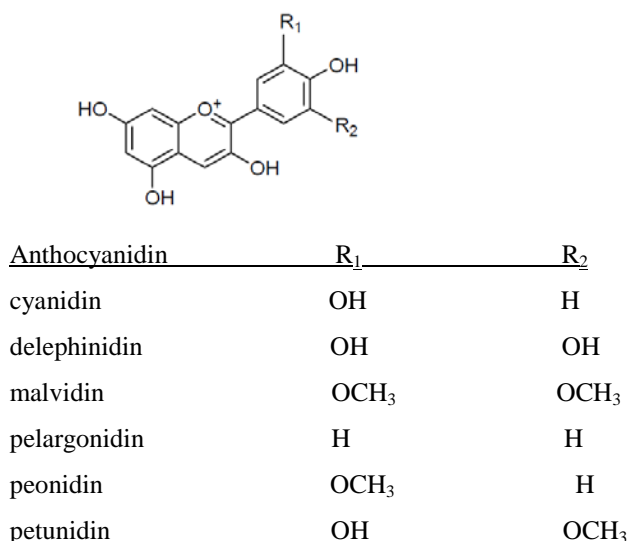


Figure 2.4 Structure of six major anthocyanidins.

The C ring of anthocyanins is fully unsaturated, so they present the most oxidized flavonoids. The anthocyanins are rather unique flavonoids; at acidic pH they have a positive charge associated with the C-ring in the form of a flavylium ion, and unlike most other flavonoids they are not negatively charged at physiological pH (Kong et al., 2003).

In aqueous medium anthocyanins undergo structural pH-dependent ring-chain tautomerism that determine their colour and stability (Mazza & Brouillard, 1987; Welch et al., 2008). A number of reversible reactions may occur while pH increases, leading to the resultant carbinol pseudo base at pH 5 and the formation of quinoidal base at pH 6-8 (Ghosh & Konishi, 2007; Passamonti et al., 2009) (Fig 2.5).

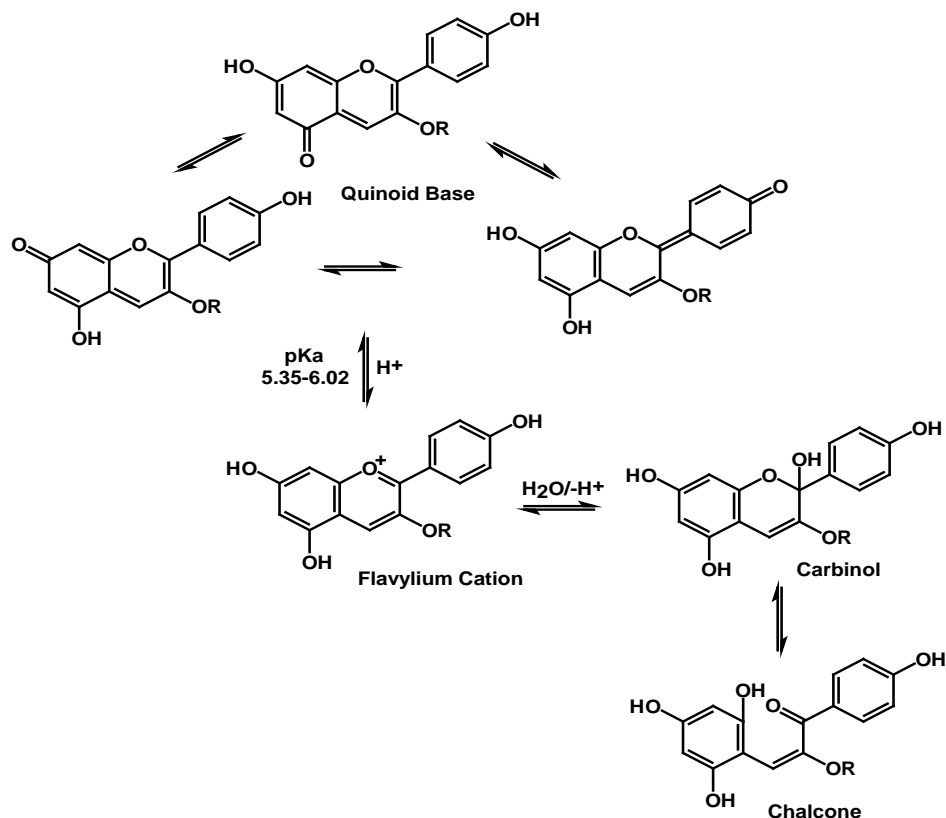


Figure 2.5 Structure of anthocyanidins in different pH. Shown are isomers of pelargonidin (aglycone $\text{R} = \text{H}$, glycone $\text{R} = \text{sugar}$). Adapted from Mazza & Buillard (1987) and Prior & Wu (2006).

The flavylium cation forms are predominantly present in acidic environments. At the more acidic pH of biological system, flavonols with a pK_a of ~ 7 (Lemańska et al., 2001) would be a mixture of neutral molecule and anion, while anthocyanins with a pK_a of 5.5-6 for the cation (Borkowski et al., 2005; Prior & Wu, 2006) would be a mixture of the neutral quinoid form with the flavylium cation. In addition to outstanding antioxidant abilities, the neutral and positive charge properties of anthocyanins compared to other flavonoids raise the reason that we are much interested in possible beneficial health properties of these compounds.

2.6.2 Antioxidant properties and protective activities against oxidative stress

Importantly, increasing interest in the potential health benefits of anthocyanins are related to efficient antioxidant activity, which is stressed from two perspectives: their free radical scavenging properties and ability to prevent radical formation by metal chelation (Kong et al., 2003). The antioxidant activity of anthocyanins and their other functions has been well studied, as well as the correlation between their antioxidant capacity and chemical structure (Welch et al., 2008). The different patterns of hydroxylation and glycosylation influence their antioxidant properties (Rice-Evans et al., 1996). Also the hydroxyl moieties of the C ring allows good metal chelation ability (Welch et al., 2008).

Anthocyanins showed much stronger antioxidant ability than most other flavonoids in the trolox equivalent antioxidant activity (TEAC) test (Rice-Evans et al., 1996). In addition, studies show that anthocyanins can have higher antioxidative activity than vitamin E, ascorbic acid, and β -carotene in certain systems (Kowalczyk et al., 2003; Rice-Evans et al., 1996; Wang et al., 1999).

Anthocyanins present high antioxidant capacity in several lipid oxidation systems, which is related to their ability to inhibit lipid peroxidation by free radical scavenging activity and metal chelating properties (Noda et al., 2002; Tsuda et al., 1996). Three cyanidin-glycoside derivatives and the aglycone cyanidin isolated from tart cherries were evaluated in a liposome system; anthocyanins inhibited lipid peroxidation and presented higher antioxidant ability than vitamin E (Wang et al., 1999). Moreover, the result suggested that the aglycone form (cyanidin) has higher efficacy than its glycosides. Studies also reported anthocyanins acted as antioxidants on human low-

density lipoprotein (LDL) against copper ion induced oxidation *in vitro* (Galvano et al., 2004).

Just like other flavonoids, anthocyanins can scavenge various oxidizing species (Noda et al., 2002). Also, they can act as scavengers of peroxynitrite; cyanidin-3-O-glucoside reduced the peroxynitrite-induced suppression of mitochondrial respiration and DNA damage in human umbilical vein endothelial cells *in vitro*, which indicated that anthocyanins may have protective effects against endothelial dysfunction and vascular failure (Serraino et al., 2003).

In addition, the protective antioxidant ability against oxidative stress has been estimated in several *in vivo* studies. Cyanidin-glucoside oral administration lowered serum TBARS formation, and protected the serum against lipid peroxidation (Tsuda et al., 1998). The *in vivo* antioxidant activity of anthocyanins also was confirmed in a model of hepatic ischemia/reperfusion (I/R) injury (Tsuda et al., 1999a). The dietary administration of cyanidin-glucoside suppressed I/R-induced indices of oxidative damage. Also consumption of an anthocyanins-rich diet improved plasma antioxidant capacity, and decreased both lipid peroxidation and DNA damage caused by vitamin E-deficiency in rats (Ramirez-Tortosa et al., 2001). All evidence listed above indicates that anthocyanins possess strong antioxidant properties against oxidative damage, and function as potent antioxidants under oxidative stress.

2.6.3 Biological activities of anthocyanins

The anthocyanins have an array of health-promoting benefits. Various studies have shown that anthocyanins have many beneficial health effects such as reducing age-associated oxidative stress, possessing anti-inflammatory properties, preventing cancer,

and protecting against vascular disease (Dai et al., 2009; Hirai et al., 2010; Kong et al., 2003; Neto, 2007; Thomasset et al., 2009b). Anthocyanins have many biological properties such as antioxidant, anti-inflammatory, antimicrobial, antiatherosclerotic and anticancer activities, and have been utilized for therapeutic purposes (Mazza et al., 2002; Mertens-Talcott et al., 2008; Min et al., 2010).

Animal investigations indicate a role for dietary anthocyanins in the prevention of age-related diseases, such as neurodegenerative disorders and cognitive decline, which suggests that anthocyanins may have effects on the ageing process of the brain (Goyarzu et al., 2004; Joseph et al., 2003). Due to evidence of anthocyanins' potential value in the treatment and prevention of oxidative stress-related diseases, these dietary bioactive constituents have been recommended as health-promoting products or nutritional supplements instead of drug products (Dai et al., 2009).

In diabetes, anthocyanins have shown promise in limiting ocular and cardiovascular complications. The anthocyanin extracts of *Vaccinium* fruits have been widely studied and used in the treatment of ocular and vascular alterations and diabetes mellitus (Ghosh & Konishi, 2007; Mills & Bone, 2000). Anthocyanin supplements have been shown to improve visual acuity, protect against retinal disorders including glaucoma and cataracts, and also be effective in diabetic retinopathy prevention (Ghosh & Konishi, 2007). In rats fed a high-fructose diet, the administration of anthocyanins prevented hypertension, cardiac hypertrophy and production of ROS, and expression of NADPH oxidase (Al-Awwadi et al., 2005).

The “French paradox”, which is the observation of a relatively low incidence of coronary heart disease in French people, has been primarily related to the regular

consumption of polyphenol-rich red wine (Lippi et al., 2010). Anthocyanins contribute predominately to the total antioxidant activities and vascular effect of red wine (Rivero-Perez et al., 2008), and so may be important contributors to the French paradox. *In vitro* laboratory studies indicate that anthocyanins may help maintain a healthy vascular system by several mechanisms. Anthocyanin extracts are effective in decreasing capillary permeability and fragility, and possess stronger anti-inflammatory and anti-oedema activities than flavonols, which suggests they are potential medical compounds against tissue inflammation or capillary fragility (Kong et al., 2003).

Effects of anthocyanins on vascular diseases include: (a) stabilizing connective tissue, (b) promoting collagen formation and (c) helping to prevent oxidative damage to blood vessels (Andres-Lacueva et al., 2005). Anthocyanins could stabilize the phospholipids of endothelial cells and improve the synthesis of collagen and mucopolysaccharides, and thus have protective effects on the endothelium of the vascular wall (Mian et al., 1977). Also, anthocyanins induce vasorelaxation, related to increased synthesis and release of endothelial nitric oxide (Mendes et al., 2003; Rivero-Perez et al., 2008).

A prospective epidemiological study indicated an inverse relationship between anthocyanin consumption and the risk of cardiovascular disease (Mink et al., 2007). Postmenopausal women (n = 34,489) involved in the Iowa Women's Health Study after 16 y of follow-up, had significant inverse associations between anthocyanin consumption, strongest among the flavonoids, and mortality of CVD and CHD after multivariate adjustment. Red wine intake was associated with a significant reduction in

CHD and CVD mortality, and a significant reduction of mortality was also related with the consumption of strawberries and blueberries.

Anthocyanins also show potential anticancer activities. Anticancer properties of anthocyanins were present in inhibiting the growth of a range of tumor cells and inducing apoptosis (Chen et al., 2005; Dai et al., 2009; Hyun & Chung, 2004).

Anthocyanins also have been investigated in clinical trials. In one trial (Thomasset et al., 2009a) 25 colorectal cancer patients were taking mirtocyan, an anthocyanin-rich standardized bilberry extract, daily for 7 days before having resection of primary tumor or liver metastases. Anthocyanin concentrations were detected in plasma, colorectal tissue and urine, and tumor tissues had decreased proliferation and increased apoptosis.

Recently, studies also showed that cyanidin and its derivatives exhibit anti-diabetic activity, which gives benefit for the prevention of obesity and diabetes (Nasri et al., 2011; Sasaki et al., 2007; Tsuda et al., 2003).

2.6.4 Bioavailability and distribution in tissues

Many studies have investigated anthocyanin absorption and metabolism both in human and experimental animals, and fruit extracts were mainly used as anthocyanin sources (Bub et al., 2001; Cao & Prior, 1999; Matsumoto et al., 2001; Mazza et al., 2002; Prior & Wu, 2006). Anthocyanins are rapidly and directly absorbed, and anthocyanin consumption resulted in a significant increase in plasma and serum antioxidant capacity (Hassimotto et al., 2008; Mazza et al., 2002).

The bioavailability and tissue distribution of anthocyanins were evaluated in a rat model using a bilberry extract that contained 15 different anthocyanins, and the results showed that the bioavailability of anthocyanins was in the range of 0.61-1.82% (0.93%

as the anthocyanin mixture) (Ichiyanagi et al., 2006). The Hassimotto group established that cyanidin glycosides absorption AUC_{0-8h} (area under the curve of concentration versus time) was $2.76 \pm 0.88 \mu\text{g hour/mL}$ and $9.74 \pm 0.75 \mu\text{g hour/g}$ for plasma and kidney after 125 mg wild mulberry extract (79% cyanidin-3-glucoside, 19% cyanidin-3-rutinoside) per kg body weight oral administration (Hassimotto et al., 2008).

In addition, anthocyanin absorption and metabolism are suggested to depend on the core phenolic aglycones and conjugated carbohydrate moieties (Ichiyanagi et al., 2006; Matsumoto et al., 2001; McGhie et al., 2003; Wu et al., 2005). Also, studies suggest that anthocyanin glycosides, unlike other flavonoids, are absorbed and distributed in their intact, unmetabolized forms (Cao & Prior, 1999; Matsumoto et al., 2001).

Anthocyanins have an affinity for tissues such as kidney and skin and can accumulate in tissues (Matsumoto et al., 2006a). Studies in rats, rabbits and pigs have shown anthocyanin absorption from the stomach and intestines to be present in plasma, liver, kidney, brain and ocular region, and unlike other flavonoids, to be excreted in urine in unmetabolized forms (Andres-Lacueva et al., 2005; Hassimotto et al., 2008; Ichiyanagi et al., 2004a; Ichiyanagi et al., 2004b; Ichiyanagi et al., 2006; Ichiyanagi et al., 2005; Kalt et al., 2008; Matsumoto et al., 2006a; Matsumoto et al., 2006b; Passamonti et al., 2005; Talavéra et al., 2005; Tsuda et al., 1999b). A summary of tissue distribution studies and the detailed methods and results are listed in Table 2.2.

Table 2.2 Previous studies reporting the presence of anthocyanins in tissues when administered via different protocols.

Model	Administration	Dose per kg body weight	Time period	Detected in tissues and body fluid	Reference
Rat	Intravenous	5mg bilberry extract (1.9 mg anthocyanins, 0.228ng cyanidin glycones) Single dose.	15 mins.	Liver, kidney, bile, plasma (1.2 μ M) urine.	(Ichiyanagi et al., 2006)
Rat	Oral	400 mg bilberries extract (153.2 mg anthocyanins, 0.01824mg cyanidin glycones). Single dose.	15 mins.	Liver (cyanidin glycones 7.54 pMol/g), kidney (cyanidin glycones 32.55 pMol/g), bile, plasma (cyanidin glycones 0.14 μ M), urine.	
Rat	Intraperitoneal	500 mg blackcurrant powder (108 mg anthocyanins). Single dose.	30 min, 1 h, 2 h, 4 h, 8 h and 24 h.	Plasma (2.30 \pm 0.76 μ g/ml (C_{max}) anthocyanins after 1h), ocular region (4.99 \pm 0.48 ng/g (C_{max}) anthocyanins after 2h).	(Matsumoto et al., 2006b)
Rat	Oral	463 mg blackcurrant powder (100 mg anthocyanins). Single dose.	30 min, 1 h, 2 h, 4 h, 8 h and 24 h.	Plasma (1.94 \pm 0.44 μ g/ml (C_{max}) anthocyanins after 30 min),ocular region (115 \pm 32 μ g/g (C_{max}) anthocyanins after 30 min).	
Rabbits	Intravenous	96.2 mg blackcurrant powder (20mg anthocyanins). Single dose.	30 min, 1 h, 2 h, 4 h, 8 h and 24 h.	Plasma (12.42 \pm 1.25 μ g/ml (C_{max}) anthocyanins after 30 min), ocular region (sclera 3.02 \pm 0.09 μ g/g, choroid 3.00 \pm 0.06 μ g/g, ciliary body 2.04 \pm 0.28 μ g/g anthocyanins after 30 min).	
Rat	Oral	152 μ Mol delphinidin-3-rutinoside from blackcurrant extract. Single dose.	1, 5, 15, 30, 60, and 120 min.	Bile, plasma (C_{max} 9.255 \pm 5.472 μ Mol/L, T_{max} of 26.3 min) (270 \pm 70 nMol after 30 min), urine.	(Matsumoto et al., 2006a)
Rat	Intravenous	7.62 μ Mol delphinidin-3-rutinoside from blackcurrant extract. Single dose.	1, 5, 15, 30, 60, and 120 min.	Bileplasma (C_{max} 0.285 \pm 0.071 μ Mol/L, T_{max} of 26.3 min), urine.	
Rat	Oral	100 mg cyanidin 3-O-beta-D-glucopyranoside extracted from blackcurrant extract. Single dose.	15, 30, 60, 120, 240, 360, and 480 min.	Plasma (maximum 0.18 μ M after 30mins), urine.	(Ichiyanagi et al., 2005)
Rat	Intravenous	2 mg cyanidin 3-O-beta-D-glucopyranoside extracted from blackcurrant. Single dose.	15, 30, 60, 120, 240, 360, and 480 min.	Plasma, urine.	
Rat	Oral	100mg delphinidin 3-O-beta-D-glucopyranoside extracted from blackcurrant. Single dose.	15, 30, 60, 120, or 240 min.	Liver, kidney, urine, plasma (0.4 μ M in 15mins.,and 30 nM after 4h)	(Ichiyanagi et al., 2004b)

Rat	Oral	100mg delphinidin 3-O-beta-D-glucoside extracted from blackcurrant. Single dose.	15, 30, 60, 120, or 240 min.	Liver, kidney, urine, plasma (appeared at 15min).	(Ichiyanagi et al., 2004a)
Rat	Gavage	0.9 mMol cyanidin 3-O-beta-D-glucoside. Single dose.	15, 30, 60, 120, or 240 min.	At 30 min, stomach (about 1.5 μ Mol/g), jejunum (about 0.14 μ Mol/g), kidney (3.20 \pm 0.26 nMol/g), liver (about 0.6 nMol/g), plasma (0.31 \pm 0.03 μ Mol/L).	(Tsuda et al., 1999b)
Rat	Gavage	125mg wild mulberry extract (79% cyanidin-3-glucoside, 19% cyanidin-3-rutinoside). Single dose.	15, 30, 60, 120, 180, 240, and 480 min.	Kidney (9.74 \pm 0.75 μ g hour/g), gastrointestinal tract (about 9mg in stomach), plasma (2.76 \pm 0.88 μ g hour/mL)	(Hassimotto et al., 2008)
Rat	Surgically induce into stomach	8 mg anthocyanin extracted from grapes. Single dose.	10 min.	Brain (192.2 \pm 57.5 ng/g), plasma (176.4 \pm 50.5 ng/mL).	(Passamonti et al., 2005)
Rat	Ad libitum	0.4 mg blueberry extract in diet. 10 weeks.	10 weeks.	Brain, plasma.	(Andres-Lacueva et al., 2005)
Rat	Ad libitum	15 mg blackberry extract (1.48mMol/kg diet anthocyanins, and > 90% cyanidin glucosides) in diet. 15 days.	sacrificed at 3 h after the beginning of the last meal.	Brain (cyanidin glucoside 0.21 \pm 0.05nMol/g, stomach (cyanidin glucoside 62.9 \pm 5.4 nMol/g), jejunum (cyanidin glucoside 485 \pm 54 nMol/g), kidney (cyanidin glucoside 2.16 \pm 0.94 nMol/g, liver (cyanidin glucoside 0.05 \pm 0.01nMol/g), urine (0.62 \pm 0.08 μ Mol/24h), plasma (0.36 \pm 0.02 μ Mol/L).	(Talavéra et al., 2005)
Pig	Ad libitum	2% blueberry powder in diet (8.5 μ Mol anthocyanins). 30 days.	30 days.	Liver (1.3 pMol/g), eyes (1.58 pMol/g), cortex (0.878 pMol/g FW), cerebellum (0.664 pMol/g FW).	(Kalt et al., 2008)

In summary, studies on the absorption and metabolism of anthocyanins have been reported mostly during the past two decades. They have suggested that anthocyanins can accumulate in tissues and are absorbed intact, and absorption can be saturated. Interest in the *in vivo* bioavailability of anthocyanins arises from evidence supporting a role for these flavonoids in human health.

However, such studies using fruit extracts as anthocyanin sources are not suitable for precise discussion of anthocyanin pharmacokinetics. Also, there is no study that compares absorption and accumulation between other flavonoids and anthocyanins.

More importantly, the question of anthocyanidin distribution and levels in subcellular organelles such as mitochondria has not been documented. With respect to oxidative stress damage, the information of mitochondria and specific tissues distribution of pure anthocyanins and their effects is critically needed.

2.7 Summary and Critical considerations

Flavonoids are considered to be promising dietary compounds with a potential beneficial roles in human health. It is suggested that flavonoids have efficiency against oxidative damage and preventing oxidative stress related diseases due to their powerful antioxidant and radical-scavenging activities. Flavonoids have been shown to be absorbed and accumulate in tissues and plasma where they could exert antioxidant activities. Experimental data have demonstrated that flavonoids can protect cell components against oxidative damage both *in vitro* and *in vivo*. Still the details on concentrations and metabolic mechanism in tissues and cells after flavonoids administration are uncertain. Also, the question remains how flavonoids enter the cells and whether they could

accumulate in subcellular organelles such as mitochondria where the antioxidant and other activities could be exerted.

Anthocyanins could provide potential pharmacological or functional food therapies against oxidative stress because of their strong antioxidant ability and unique characteristics. The anthocyanins are unique among the flavonoids in not being negatively charged at physiological pH, and show especially efficient antioxidant activities and free radical scavenging properties. The *in vitro* antioxidant activities of anthocyanins and protective mechanism among their structural properties are highly studied recently. The antioxidant efficacy *in vivo* of anthocyanins has been less thoroughly documented, and further confirmation is still needed. Also, with regards to anthocyanins there are still fewer studies compared to studies of other flavonoids.

Lipophilic cations can be used to target an attached antioxidant to mitochondria (Armstrong, 2008), and in principle anthocyanins may have a positive charge at the acidic interface of the mitochondrial membrane. This raises the question of whether anthocyanins may exert protective effects against the oxidative damage implicated in mitochondria.

Many experiments indicate anthocyanins are rapidly absorbed, and can reach the tissues, and anthocyanins consumption resulted in significant increases in plasma and serum antioxidant capacity. For further discussion on *in vivo* function of anthocyanins in system, it is critical to know the physiological uptake and distribution of each anthocyanin in tissues and perhaps especially in mitochondria. However, there are no data on the potential ability of anthocyanins to reach and affect mitochondria. For a better understanding of their potential health effects, this information is both essential and

necessary. The main goal of this research was to provide detailed evidence about anthocyanin uptake by mitochondria and distribution in tissues and mitochondria that may relate to their health benefits in preventing oxidative stress and age-related disease.

3. HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

The anthocyanins, to a greater extent than other flavonoids will be driven by an electrical potential mechanism into mitochondria, where they will have protective effects. Thus, during treatment with pure anthocyanins *in vitro* or *in vivo*, mitochondria isolated from rat liver would show an increased content of anthocyanins; and decreased susceptibility of mitochondria to oxidative damage.

3.2 Objectives

The overall objective of my thesis was to compare the mitochondrial uptake and antioxidant activities of selected flavonoids after delivery *in vitro* and *in vivo*. Cyanidin, quercetin and their 3-glucosides were studied in rats and isolated rat liver mitochondria.

Specific objectives were:

1. *In vitro*, to determine the relative mitochondrial loading of the selected flavonoids, the involvement of mitochondrial membrane potential in uptake, and the impact on mitochondrial respiration and susceptibility to oxidative stress.
2. *In vivo*, to analyze and compare the distribution and protective effects of intravenously delivered flavonoids on tissues and mitochondria.

4. MATERIALS AND METHODS

4.1 Experimental animals and chemicals

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. For all experiments, adult male Wistar rats (250-300 g body weight) were used and purchased from the University of Saskatchewan Animal Resource Centre. Animals were housed in a temperature- ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity- ($50 \pm 10\%$) controlled facility on 12h light/dark cycles (0700 h – 1900 h) in accordance with the guidelines of the Canadian Council on Animal Care. The rats were fasted overnight prior to the experiment.

There were four selected flavonoids tested in this study: cyanidin (CY), cyanidin-3-O-glucoside (C3G), quercetin (QU) and quercetin-3-glucoside (Q3G). Chemicals, unless specified, were purchased from Sigma-Aldrich (St. Louis, MO). Cyanidin chloride and kuromanin chloride (cyanidin-3-O-glucoside chloride) were obtained from Extrasynthese SA (Z.I Lyon Nord. France).

4.2 In vitro study

4.2.1 Mitochondrial uptake measurements

4.2.1.1 Mitochondria isolation

Mitochondria were isolated from rat liver following humane euthanasia (isoflurane anesthesia and decapitation) and rapid removal of organs. Briefly, freshly excised liver

was cut into small pieces and homogenized using a glass-teflon homogenizer with an ice-cold isolation medium containing 250 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.2 (~4 mL/g tissue). The homogenate was then centrifuged at 1000 g for 8 min at 4°C. The supernatant was collected and centrifuged at 10,000 g for 10 min at 4°C. The pellet was resuspended in washing medium containing 250 mM sucrose, 10 mM HEPES and 0.1 mM EGTA (pH 7.2) and again centrifuged at 10,000 g for 10 min at 4°C. The final pellets of mitochondria were suspended in 2 mL of isolation medium without EGTA (pH 7.2).

4.2.1.2 Mitochondria treatment/loading conditions

Test flavonoids at 125 μ M were incubated with fresh RLM (1 mg/mL of protein) in 1 mL sucrose-HEPES buffer (250 mM sucrose, 10 mM HEPES, pH 7.2) in the presence and absence of a respiratory substrate (succinate 5 mM), and an uncoupler (CCCP, 2 μ M) in room temperature for 10 min. The mitochondria were then centrifuged at 15,000 g for 10 min at 4 °C in the presence or absence of 2 mg/mL BSA, which adsorbs flavonoids, to determine the amount of flavonoids which entered the mitochondria compared to that which adhered to the surface. CY, C3G, and QU stock solutions (25 mM) were prepared in ethanol (with 0.1% HCl), and Q3G was in methanol (with 0.1% HCl). The flavonoids stock solutions were diluted in sucrose-HEPES buffer before use. A control incubation of RLM in the absence of added flavonoid was also performed. The mitochondrial pellets were stored in the -20°C freezer for later analysis. All incubations were done in duplicate.

4.2.1.3 Spectrophotometric analysis of flavonoid uptake

Initially, 125 μ M test flavonoid with reaction medium was scanned at room temperature in the wavelength range 400-650 nm (CY and C3G) and 260-500 nm (QU and Q3G) by a Beckman DU 640 spectrophotometer with temperature control. After

mitochondrial loading, the supernatant was rescanned to measure the content of flavonoids. The uptake of flavonoid by fresh RLM was indicated by the difference of absorbance between the initial reading and the supernatant reading (i.e. loss of absorbance). The absorbance wavelength chosen in calculation for CY was 576 nm, for QU was 376 nm, and for C3G and Q3G were 562 nm and 365 nm respectively.

4.2.1.4 HPLC analysis of flavonoid uptake

Quantification of flavonoids in mitochondrial pellets was performed on a Waters Alliance 2695 HPLC system fitted with a Waters 2996 photodiode array detector and a Waters Fraction Collector III. The HPLC system was controlled by Empower Software.

The analysis was carried out on a 250 mm×4.6 mm VARIAN Inertsil C₈ reverse-phase column (5 µm) at 25 ± 1°C. Flavonoid content detection was achieved spectrophotometrically with monitoring at wavelengths of 528 (CY), 520 (C3G), 374 (QU), and 365 (Q3G) nm.

Two different mobile phase systems were used in the flavonoids analysis. Both mobile phase systems contained a combination of polar solvent A and a less polar solvent B. HPLC analysis methods in this study are shown in Table 4.1. All solvents were HPLC grade (Aldrich-Sigma), and water was purified via a Milex Q-plus system (Millipore). The elution was performed using a solvent gradient system, the mobile phase flow rate was 1.2 mL/minute and the sample injection volume was 30 µL.

Table 4.1 Summary of flavonoid HPLC analysis profiles.

HPLC profiles for selected flavonoids	Mobile phases solvents	Elution profiles
Cyanidin	A: water+1% formic acid B: methanol +0.1% formic acid	0 - 0.5 min, 5% B (v/v); 0.5 - 20 min, linear gradient from 5 to 60% B (v/v); 20 - 22 min, linear gradient from 60 to 95% B (v/v); 22 - 25 min, isocratic elution with 95% B (v/v); 25 - 28 min, linear gradient from 95 to 5% B (v/v); 28-30 min, isocratic elution with 5% B (v/v).
Cyanidin-3-glucoside	A: water+1% formic acid B: methanol +0.1% formic acid	0 - 0.5 min, 5% B (v/v); 0.5 - 15 min, linear gradient from 5 to 60% B (v/v); 15 - 17 min, linear gradient from 60 to 95% B (v/v); 17 - 20 min, isocratic elution with 95% B (v/v); 20 - 23 min, linear gradient from 95 to 5% B (v/v); 23 - 25 min, isocratic elution with 5% B (v/v).
Quercetin	A: water+0.1% formic acid B: methanol +0.1% formic acid	0 - 0.5 min, 10% B (v/v); 0.5 - 10 min, linear gradient from 5 to 60% B (v/v); 10 - 20 min, isocratic elution with 60% B (v/v); 20 - 23 min, linear gradient from 60 to 10% B (v/v); 23 - 25 min, isocratic elution with 10% B (v/v).
Quercetin-3-glucoside	A: water+0.1% formic acid B: methanol +0.1% formic acid	0 - 0.5 min, 10% B (v/v); 0.5 - 10 min, linear gradient from 5 to 60% B (v/v); 10 - 20 min, isocratic elution with 60% B (v/v); 20 - 23 min, linear gradient from 60 to 10% B (v/v); 23 - 25 min, isocratic elution with 10% B (v/v).

The mitochondria pellets after incubation with the flavonoids and centrifugation (with and without added BSA) were resuspended in methanol with 0.1% HCl (except with QU in ethanol with 0.1% HCl). The samples were vortexed 15 seconds and left to stand for 10 min at room temperature, and then centrifuged at 15000g for 10 min at 4 °C. The supernatants were filtered (0.2 µm pore size) before injecting onto the HPLC system. All samples were analyzed in duplicate, and standards were in triplicate. The flavonoid standards were employed for retention time and quantification. Calibration equivalents of spectral peak area versus analyte concentration were plotted from various standard concentrations. Calibration curves of these standards had correlation coefficients of 0.99 or greater.

For C3G there was a slight shoulder present in the HPLC chromatograms (Appendix B). In order to determine the possible reason, the 15µM and 1.5µM C3G standard solutions were also analyzed by mass spectrometry (presented in Appendix C) which showed that the shoulder was likely due to different isomers.

4.2.2 Mitochondrial respiration rate measurements

Respiration rates were measured with a Clark-type electrode (Oxytherm System; Hansatech Instruments Ltd, Norfolk, England) in a water-jacketed glass chamber with magnetic stirring. The respiration chamber was kept at 30°C. Oxygen uptake measurements were carried out in 1 mL respiratory buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES, 2 mM KH₂PO₄/K₂HPO₄, 1 mM MgCl₂, pH 7.2), with succinate (5mM) and respiratory cocktail (5 mM each of malate, glutamate, pyruvate and α-ketoglutarate) as substrates. Initially, fresh RLM (3 mg protein) were added into the reaction medium, and a conventional respiratory experiment with transitions from state

4 to 3 was performed (Estabrook, 1967).

The trace of oxygen consumption was recorded in the absence (respiration state 4) and in the presence (respiration state 3) of 250 nmol ADP to determine the respiratory control ratio (RCR) and ADP/O ratios. The effect of 125 μ M flavonoids on state 4 respiration was measured to test for inhibitory or uncoupling effects.

4.2.3 Mitochondrial ROS generation

The effect of flavonoids on antimycin-stimulated mitochondrial ROS generation was measured with the cell permeable fluorogenic probe 2',7'-dichlorofluorescein diacetate (DCFDA). ROS generation was measured both in RLM membranes incubated in the presence of added flavonoids (125 μ M) and in the RLM pre-loaded with flavonoids (described in section 4.2.1.2). The RLM membranes were prepared by re-harvesting (15,000 g for 10 min at 4°C) frozen-thawed RLM.

To measure ROS generation RLM membranes or pre-loaded RLM (1 mg/mL of protein) were incubated in respiration buffer with 2.5 mM succinate and 2.5 mM respiratory substrate cocktail, 3 μ M antimycin and 5 μ M DCFDA, at 30°C with shaking for 30 min. After incubation, the samples were centrifuged at 15,000 g for 10 min at 4 °C. The fluorescence of aliquot supernatants was measured using a Biotek plate reader at 485/20 excitation and 530/25 emission with a sensitivity of 45. The control tests (mitochondria only, and with CCCP) were also performed at the same time.

4.2.4 Lipid peroxidation assay with flavonoids treatments

The effect of flavonoids, added to the incubation medium or present in the mitochondria after loading, on lipid peroxidation induced by t-butyl hydroperoxide was determined by the formation of thiobarbituric acid reactive substances (TBARS). The

TBARS assay was adapted from the methods of (Ohkawa et al., 1979) and (Fraga et al., 1988) with some modifications. Basically, the method analyzes reactive aldehydes such as malondialdehyde (MDA) generated under the test conditions. At low pH and high temperature, TBA reacts with such aldehydes and yields a fluorescent product. Commonly, the formation of TBARS can be detected by absorbance because of its pink chromophore with an absorption maximum around 532 nm. In this study because of the absorption of anthocyanins at 532 nm, TBARS quantification employed the fluorescence method (Fraga et al., 1988) measuring at 485/20 excitation and 540/25 emission with a sensitivity of 80. The TBARS assays were performed simultaneously with different MDA concentration standards (prepared from tetraethoxypropane by acid hydrolysis) to establish the MDA equivalents standard curve.

For the experiments with flavonoids added to the incubation medium, RLM membranes were resuspended in 1.15% KCl solution, and the protein content was measured. Resuspending in 1.15% KCl avoids the use of sucrose, which at high concentrations gives a false positive in the TBARS assay. The RLM membranes (1 mg/mL of protein) were then added in a suitable volume of 50 mM potassium phosphate buffer (pH 7.2), with selected flavonoids (125 μ M) and incubated at 37°C for 60 min with 1 mM *t*-buOOH.

For the experiments with flavonoid pre-loaded mitochondria, RLM were preloaded with flavonoid using the loading method 4.2.1.2 in the absence of substrates. The RLM pellets after pre-loading and centrifugation were then resuspended (at 1 mg/ml of protein) in the 50 mM potassium phosphate buffer (pH 7.2), and incubated at 37°C for 60 min with 1 mM *t*-buOOH.

To measure TBARS, aliquots from the incubations were mixed with 4 times the volume of incubation solution containing 8.1% SDS, 20% acetic acid (pH 3.5), 0.8% TBA and 0.02% BHT and heated at 95°C for 60 min. The TBARS products were extracted into an equal volume of butanol/pyridine (v: v.15:1). The fluorescence of the butanol layer was measured as described above.

4.2.5 Protein measurement

For all of the mitochondrial and tissue samples in study I and II, protein contents were measured by the biuret assay. Sample aliquots were incubated with 9 times the volume of biuret reagent (32 mM potassium sodium tartrate, 12 mM copper sulfate, 30 mM potassium iodide, 0.2 M NaOH) for 20 min at room temperature. Sample absorbance was then measured at 550 nm. Calibration curves with correlation coefficients of 0.99 or greater were established using BSA as the standard.

4.3 In vivo study

4.3.1 Experimental animals and treatments

Twenty five male Wistar rats (250-300 g body weight) were assigned randomly into 5 groups (n = 5/group) in this part of study.

Group 1: - control group – intravenous administration of vehicle.

Group 2: - intravenous administration of CY

Group 3: - intravenous administration of C3G.

Group 4: - intravenous administration of QU.

Group 5: - intravenous administration of Q3G.

Cyanidin, quercetin or their 3-glucosides were administered into the rat tail vein to give a dose of 7.6 $\mu\text{mol/Kg}$ body weight according to Matsumoto's studies (Matsumoto et al., 2006a; Matsumoto et al., 2006b). The rats were fasted overnight prior to injection.

The stock flavonoid solutions at 7.6 mM were prepared in 50% polyethylene glycol (PEG) 400 as the vehicle (Neervannan, 2006), and directly injected into tail vein (under isoflurane anesthesia) according to body mass. One group was treated as control and received 50% PEG 400 instead of flavonoids. The rats were returned to their cages and provided with water but were not fed.

After 30 min, which is a point after which more than 80% of an intravenous dose of anthocyanin (delphinidin-3-rutinoside) was cleared from plasma (Matsumoto et al 2006a), a blood sample was collected from the heart under anesthesia into heparin-containing tubes, and was centrifuged at 4000 g for 8 min at 4°C to collect the plasma. The rat body was perfused transcardially with cold phosphate buffered saline (PBS) for 2-3 min at a rate of 52 mL/min to remove the blood in organs, and the liver, heart, kidney and brain were removed. Half of the liver and the other organs were well washed, wrapped in aluminum foil, frozen in liquid nitrogen, and stored in a -80°C freezer. RLM were immediately isolated (method as in 4.2.1.1) from half of the liver. The RLM, post-mitochondrial supernatant, and plasma samples, were stored at -80°C for further analyses.

4.3.2 HPLC analysis of flavonoids distributed in tissues, mitochondria and plasma

All HPLC analyses in this research section were performed on an Agilent Technologies 1100 Series HPLC system (Agilent Technologies Canada Incorporated, Mississauga, ON). The HPLC system consisted of a binary pump (model G1310A), a solvent degasser (model G1379B), an autosampler (model G1367B), a column compartment (model G1316A), and a multi wavelength detector (MWD; model G1365D). The analysis was carried out with the same column and test condition as in

4.2.1.4. The HPLC analysis profiles were as in Table 4.1. The mass spectra of the flavonoid standards were employed in sample flavonoid identification and quantification. Calibration curves using standards were employed. The extraction recovery rates of flavonoids from tissues, RLM and plasma were also performed by adding known quantities of flavonoids to control samples.

4.3.2.1 Tissue sample extraction

All tissue samples were crushed into a powder under liquid nitrogen using a Bessman tissue pulverizer (BioSpec, Bartlesville, OK) before the extraction process. The protocol of flavonoids extraction from tissues was designed according to several *in vivo* studies (Miniati, 2007; Talavéra et al., 2005). Briefly, a portion of frozen tissue powder (approximately 50 mg) was homogenized in an appropriate volume of methanol with 1% formic acid at a ratio of 9 mL to 1 g tissue. Samples were homogenized using a Potter-Elvehjem (glass-Teflon) homogenizer. The supernatant was collected after centrifuging at 4000 g for 8 min at 4°C. The pellet was re-extracted with methanol with 1% formic acid at 4 mL per g of tissue and centrifuged as above. All of the extracts obtained above were combined and evaporated to dryness using rotary evaporation under vacuum ($\leq 30^{\circ}\text{C}$). The dried tissue extracts were then redissolved in methanol with 0.1% HCl (QU in ethanol with 0.1% HCl) and centrifuged at 12,000 g for 5 min at room temperature. The supernatants were filtered (0.2 μm pore size) before being applied to the HPLC system.

4.3.2.2 Mitochondria extraction

RLM samples (20 mg protein) were centrifuged at 15,000 g for 10 min at 4°C, the pellets were resuspended and homogenized in methanol with 0.1% HCl (QU in ethanol

with 0.1% HCl), followed by centrifugation as above. The supernatants were filtered (0.2 µm pore size) before being injected into the HPLC system.

Also in this study, we attempted flavonoid analysis in post-mitochondrial supernatants. One 50 mL post-mitochondrial supernatant was chosen from each flavonoid group, and placed in freeze-drying chambers until dry (~ 24 hours). The samples were extracted with 2 mL methanol containing 1% formic acid; the extracts were then evaporated to dryness using rotary evaporation under vacuum ($\leq 30^{\circ}\text{C}$). The dried tissue extracts were then redissolved in methanol containing 0.1% HCl (QU in ethanol containing 0.1% HCl) and centrifuged at 12,000 g for 5 min at room temperature. The supernatants were filtered (0.2 µm) before being applied to the HPLC system. However, flavonoid levels in these samples were not detectable.

4.3.2.3 Plasma extraction

A 500 µL portion of plasma was adjusted to pH 2.5 with 150 µL of 1 M potassium dihydrogen phosphate solution and 15 µL 85% phosphoric acid, and then 2.5 mL acetonitrile was added and the samples were vortex mixed for 1 min and centrifuged at 3500 g for 10 min at 5°C (Miniati, 2007). The supernatant liquors were evaporated to dryness using rotary evaporation at room temperature. The sample residues were extracted and HPLC-MS analysis was performed as described above.

4.3.3 Endogenous TBARS of tissues

Weighed samples (about 50 mg) of the frozen powdered tissues were homogenized with 200 µL RIPA buffer (50mM Tris, 150 mM KCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% EDTA, pH 7.4), and after incubation at room temperature for 30 min to allow dissolution of the tissues, were centrifuged at 10,000 g for 10 min at 4°C.

The supernatants were heated with 4 times the volume of incubation solution containing 8.1% SDS, 20% acetic acid, 0.8% TBA and 0.02% BHT at 95°C for 60 min, and TBARS analysis was performed (as in 4.2.4). The protein measurement was also performed on the homogenates.

4.3.4 Susceptibility of RLM to TBARS formation *ex vivo*

Frozen-thawed RLM were reharvested (15,000 g for 10 min at 4°C), resuspended in 1.15% KCl solution, and protein contents were measured. The RLM were then incubated at 1 mg protein/mL in 50 mM potassium phosphate buffer (pH 7.2) with 1 mM *t*-buOOH at 37°C for 60 min. The TBARS tests were completed as described previously (4.2.4).

4.4 Statistical Analysis

Results are presented as means \pm SEM. Data were analyzed by one-way analysis of variance with Tukey's Honestly Significant Differences (HSD) *post hoc* analysis, and differences were considered statistically significant at $p < 0.05$.

5. RESULTS

5.1 In vitro study

5.1.1 Flavonoid accumulation in mitochondria *in vitro* study

Comparing of the uptake of the different flavonoids in freshly prepared RLM using both the spectrophotometric method (Figure 5.1) and HPLC analysis of the mitochondrial pellet after centrifugation (Figure 5.2) showed large differences between the flavonoids. The two methods gave very good agreement, although the spectrometric method gave higher values. Representative spectrometric scans are presented in Appendix A, and representative HPLC chromatograms are presented in Appendix B.

Notably, the uptake of CY in isolated mitochondria was significantly higher than the other three flavonoids under all experimental conditions examined ($p < 0.05$) (Figures 5.1 and 5.2). The spectrophotometric method showed 78% uptake of 125 μM CY compared to $<33\%$ for the other flavonoids. The HPLC measurements in the mitochondrial pellets showed 67% uptake of 125 μM CY compared to $<19\%$ for the other flavonoids. In addition, both methods indicated there was a much lower uptake of C3G compared to CY ($p < 0.05$). Also with quercetin the spectrophotometric results showed significantly ($p < 0.05$) and the HPLC results showed a tendency ($p = 0.076$) towards lower absorption of Q3G compared to its aglycone QU. The absorption of C3G was not significantly different from Q3G as determined by both measurement techniques.

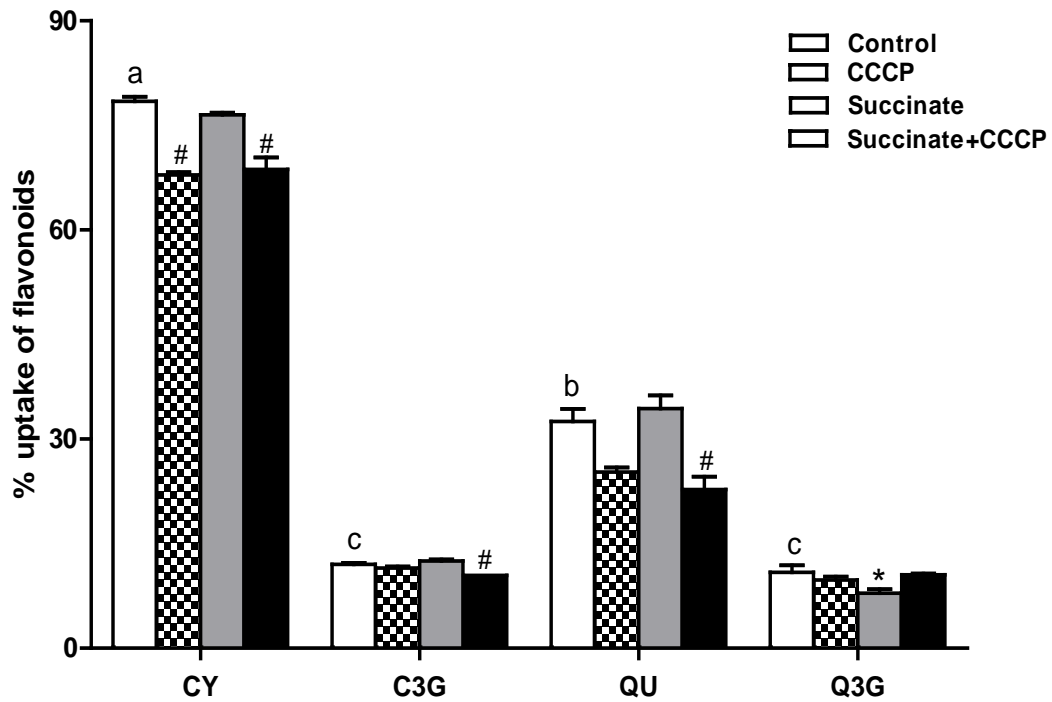


Figure 5.1 Spectrophotometric analysis of mitochondrial flavonoid uptake.

Percent (%) uptake of flavonoids (125 μ M) by RLM (1 mg protein) was determined in 1 mL sucrose-HEPES buffer (pH 7.2) incubated at room temperature in the presence and absence of a respiratory substrate (succinate), and an uncoupler (CCCP). The control was the flavonoids incubated with fresh RLM in the absence of other additions. The amount of flavonoid remaining in the buffer was measured by the spectrometric method in RLM supernatants after centrifugation. Shown are the results from samples treated with BSA prior to centrifugation. Representative scans of the medium before incubation with RLM and after centrifugation are shown in Appendix A. Results are expressed as means \pm SEM (n=3 mitochondrial preparations), with each experiment performed in triplicate.

^{ab} Different letters show significant differences between flavonoids at $p < 0.05$.

Bars with the same letters indicate no significant difference at $p < 0.05$.

* Significantly different from the corresponding control without succinate. $p < 0.05$;

Significantly different from the corresponding condition without CCCP. $p < 0.05$.

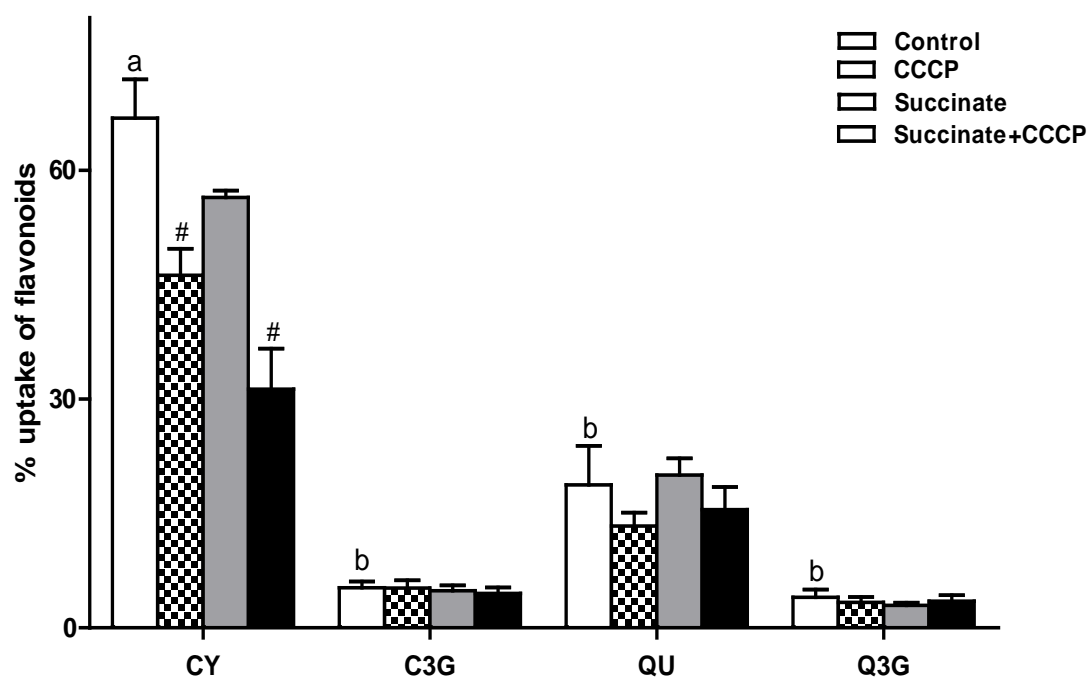


Figure 5.2 HPLC analysis of mitochondrial flavonoid uptake. Percent (%) uptake of flavonoids (125 μ M) by RLM (1 mg protein) was determined in 1 mL sucrose-HEPES buffer (pH 7.2) incubated at room temperature in the presence and absence of a respiratory substrate (succinate), and an uncoupler (CCCP). The control was the flavonoids incubated with fresh RLM in the absence of other additions. The amount of flavonoid remaining in the buffer was measured by the HPLC method in RLM pellets after being treated with BSA and centrifuged. Results are expressed as means \pm SEM (n=3 mitochondrial preparations), with each experiment performed in triplicate.
^{ab} Different letters show significant differences between flavonoids at $p < 0.05$.

Bars with the same letters indicate no significant difference at $p < 0.05$.

[#] Significantly different from the corresponding condition without CCCP. $p < 0.05$.

For simplicity, the detailed statistical results of flavonoids with succinate and/or CCCP are not shown in the graphs. However, with CCCP or succinate present, not only was CY uptake significantly higher than C3G, but QU uptake was significantly higher than Q3G ($p < 0.05$).

With regard to the effects of the respiratory uncoupler (CCCP), only with CY did CCCP give significant differences observed, inhibiting uptake by 31.31-46.23 % in the presence or absence of succinate (Figures 5.1 and 5.2). With regard to the effect of

substrate (succinate), no significant differences were found with succinate compared to the condition without succinate except for a significant slight effect on Q3G uptake measured by the spectrophotometric method (Figure 5.1).

After incubations of RLM with flavonoids, centrifugations were conducted in the presence and absence of BSA to determine how tightly the flavonoids were bound or taken up. No statistically significant differences were observed between the presence and absence of BSA for any of the four flavonoids (Figure 5.3).

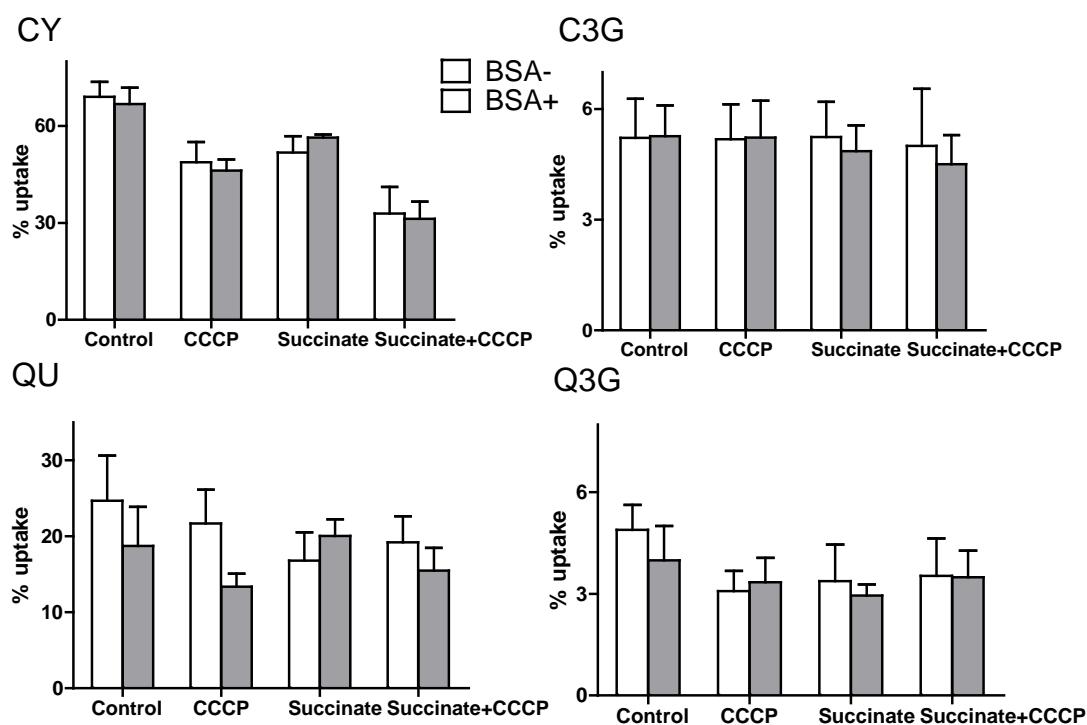


Figure 5.3 Resistance of mitochondrial bound/uptaken flavonoids to removal by BSA. Percent (%) uptake of flavonoids (125 μ M) by RLM (1 mg protein) was measured by the HPLC method. Experiments were conducted in 1mL sucrose-HEPES buffer in the presence and absence of a respiratory substrate (succinate), and an uncoupler (CCCP) and were compared between with BSA and without BSA added before centrifugation. The control was the flavonoids incubated with fresh RLM in the absent of other additions.

Results are expressed as means \pm SEM (n=3 mitochondrial preparations), with each experiment performed in triplicate. There were no significant differences ($p < 0.05$) observed between treatments with and without BSA for the four flavonoids.

5.1.2 Flavonoid effects on mitochondrial respiration

Fresh RLM were examined for their oxygen consumption response to the four flavonoids and ADP (Table 5.1). After adding ADP to the reaction medium, the oxygen consumption increased to 4-5 times that of the control, which indicated the RLM used in the test were active and well functional. The respiratory control test was performed for each mitochondrial preparation in order to confirm the mitochondrial integrity. Measurements of the effects of 125 μ M flavonoids on state 4 respirations did not show any significant effects.

Table 5.1 Respiratory parameters of mitochondria treated with ADP or flavonoids. Fresh RLM (1 mg protein) were examined for their oxygen consumption response to the flavonoids (125 μ M) and ADP (250 nmol) in 1 mL respiratory buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES, 2 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 1 mM MgCl_2 , pH 7.2), with succinate (5mM) and respiratory cocktail (5 mM each of malate, glutamate, pyruvate and α -ketoglutarate) as substrates. The control was the fresh RLM in the absent of other additions. Results are expressed as means \pm SEM (n=2 mitochondrial preparations), with each experiment performed in duplicate. Rates were not significantly different from the control at $p < 0.05$ (except ADP treatment).

Test conditions	Oxygen consumption rate (nmol O_2 / min)
Control	14.54 \pm 0.58
ADP	68.01 \pm 5.33
CY	18.32 \pm 0.81
C3G	14.70 \pm 0.66
QU	18.74 \pm 0.26
Q3G	19.78 \pm 0.22

5.1.3 Protective effects of select flavonoids on antimycin-stimulated mitochondrial ROS generation

The ROS generation measurements were calculated as fluorescence units per mg mitochondrial protein, and expressed as percentage of the antimycin-stimulated

condition, which was marked as the 100% value. The antimycin-stimulated ROS generation level was 724 ± 132 fluorescence units per mg mitochondrial protein in the directly-incubated (ie. not previously incubated in the preloading condition) RLM, and 2806 ± 193 fluorescence units per mg mitochondrial protein, in the preincubated and reharvested condition.

Results showed that RLM incubated or preloaded with each of the four flavonoids significantly decreased antimycin-stimulated ROS generation (to $< 29\%$ and $< 56\%$, respectively); and at similar amounts, during respiration ($p < 0.05$) (Figure 5.4).

Although not as effective as adding flavanoids ($125 \mu\text{M}$) to the incubations; preloading flavonoids inhibited antimycin-stimulated ROS generation to a level less than the controls (without antimycin). When incubated together with RLM, CY showed a slightly lesser effect on the ROS generation than other flavonoids ($p < 0.05$). Whereas, preloaded CY was found to be the most protective (albeit not statistically significant) treatment.

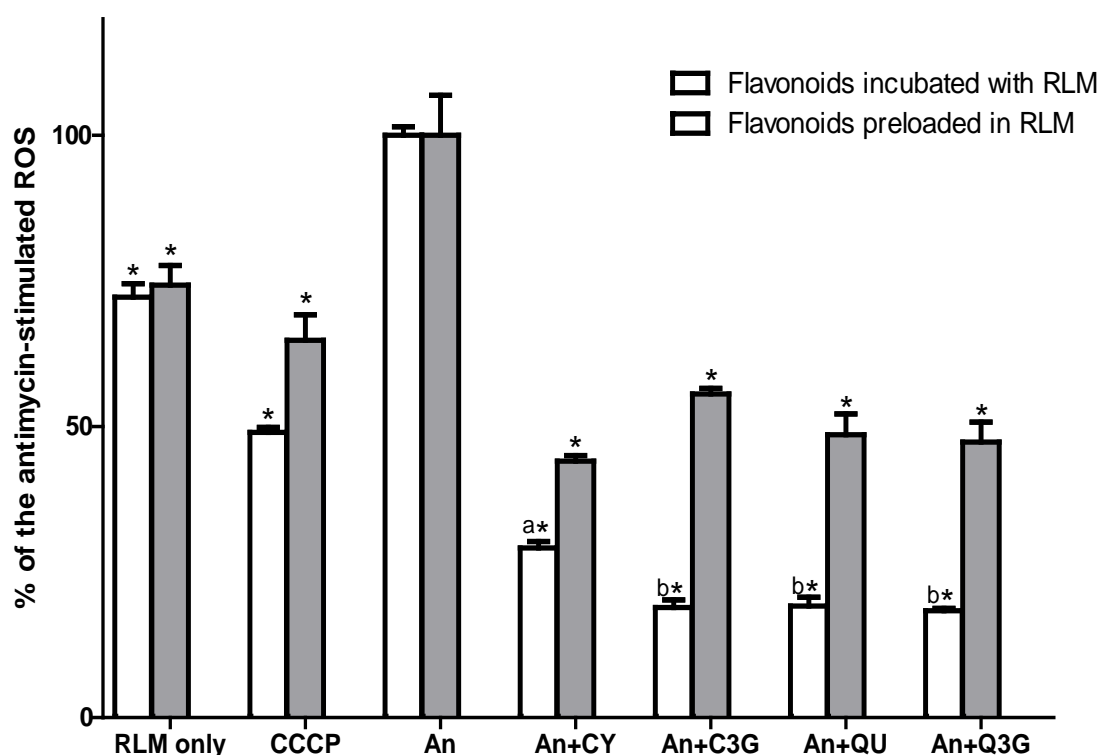


Figure 5.4 Effects of incubation with flavonoids or pre-loading of flavonoids on antimycin-stimulated mitochondrial ROS generation. Mitochondrial ROS generation was measured during incubation with respiratory substrates in two test conditions: RLM incubated with added flavonoids, and RLM with flavonoids-preloaded from the experiments shown in Figures 5.1 and 5.2. Flavonoids (125 μ M) incubated with RLM (1 mg/mL of protein) or flavonoid-preloaded RLM (1 mg/mL of protein) were incubated in 500 μ L of respiration buffer with 2.5 mM succinate and 2.5 mM substrate cocktail, 3 μ M antimycin (An) and 5 μ M DCFDA at 30°C for 30 min. Results are expressed as means \pm SEM (n=3 mitochondrial preparations), with each experiment performed in triplicate.

* Significantly different from the corresponding antimycin-stimulated condition. p < 0.05;

^{ab} Different letters show significant differences between flavonoids at p < 0.05.

5.1.4 Flavonoids effects on lipid peroxidation

The susceptibility of RLM to lipid peroxidation induced by *t*-buOOH was determined by the TBARS assay (Figure 5.5). The mitochondria-only and *t*-buOOH-induced TBARS levels were 0.65 ± 0.03 nmol and 2.19 ± 0.04 nmol MDA equivalents/mg mitochondrial protein in the directly-incubated (ie. not previously

incubated in the preloading condition) RLM, and 0.17 ± 0.001 nmol and 1.45 ± 0.08 nmol MDA equivalents/mg mitochondrial protein in the preincubated and reharvested condition. The results are expressed as percentage of the *t*-buOOH-induced TBARS condition.

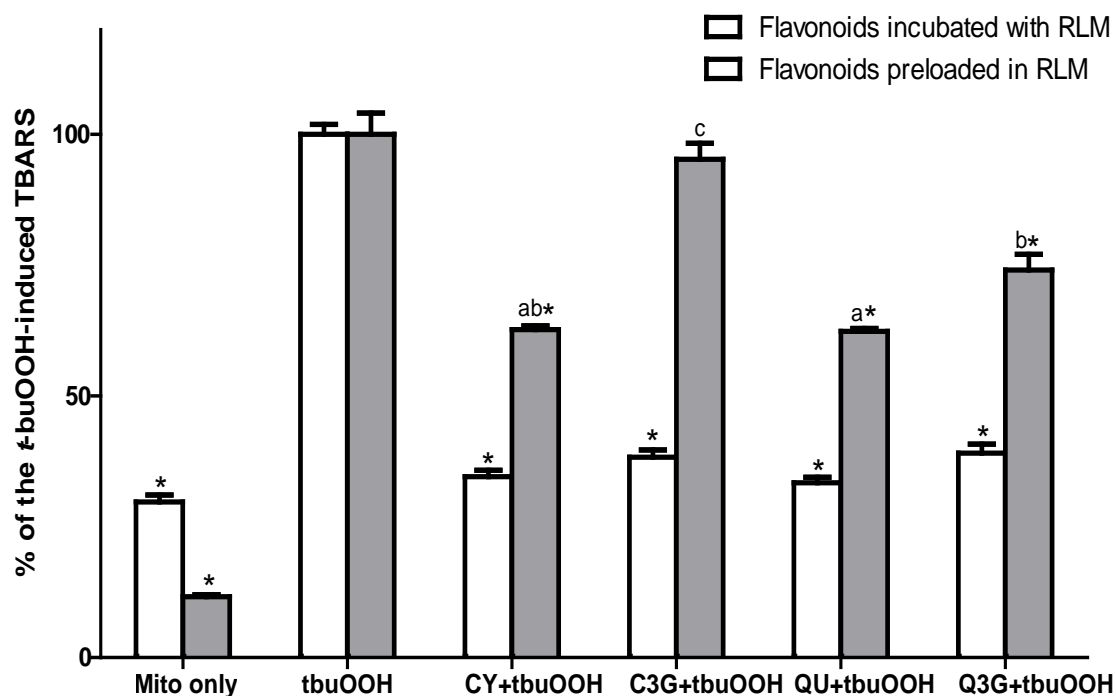


Figure 5.5 *t*-buOOH-induced TBARS formation for mitochondria incubated or preloaded with flavonoids (125 μ M). Mitochondrial lipid peroxidations were measured during incubation with *t*-buOOH (tbuOOH) in two test conditions: RLM incubated with 125 μ M added flavonoids and RLM with flavonoids-preloaded (from the experiments shown in Figures 5.1 and 5.2). The RLM (at 1 mg/mL of protein) were incubated in 50 mM potassium phosphate buffer (pH 7.2) with 1 mM *t*-buOOH at 37°C for 60 min, after which the formation of TBARS was assayed. Results are expressed as means \pm SEM (n=3 mitochondrial preparations), with each experiment performed in triplicate.

* Significantly different from the corresponding *t*-buOOH condition. $p < 0.05$.

^{ab} Different letters show significant differences between flavonoids at $p < 0.05$.

When incubated with RLM, each of the four flavonoids (125 μ M) almost completely inhibited *t*-buOOH-induced lipid peroxidation (TBARS formation). With preloaded flavonoids, the aglycone flavonoids (CY and QU) gave better protection

compared to their glucoside ($p < 0.05$) (Figure 5.5), which coincided with greater loading (Figures 5.1 and 5.2).

5.2 In vivo study

5.2.1 Flavonoids distribution in plasma, tissues and mitochondria

5.2.1.1 Flavonoids distribution in plasma

In plasma 30 min after administration, the concentration of QU was found to be similar to its glucoside and was significantly higher than CY and C3G ($p < 0.05$) (Figure 5.6). The results indicated that the flavonols remained more in plasma than the anthocyanins, and both flavonoids presented to an equal extent with their glucosides.

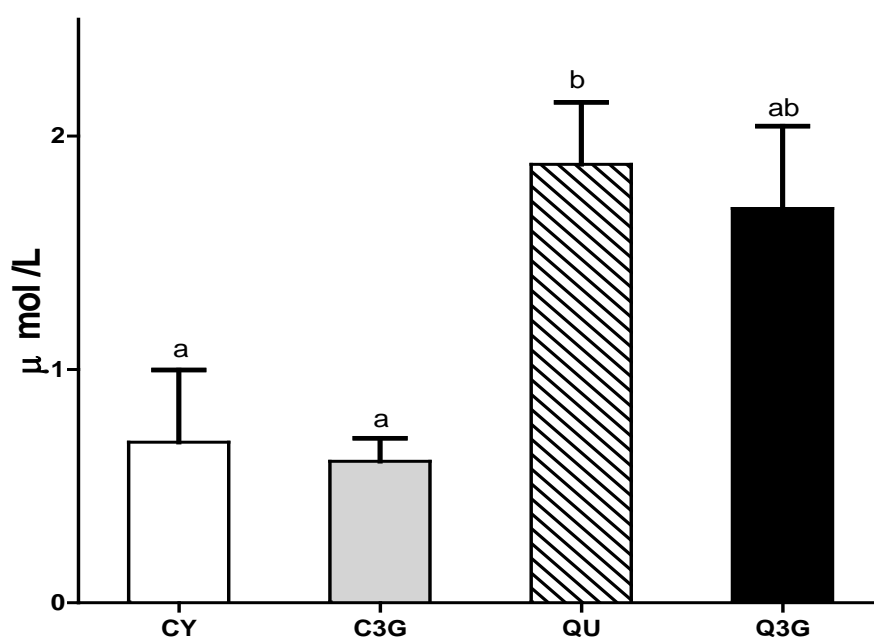


Figure 5.6 Distribution of flavonoids in plasma. CY, QU and their 3-glucosides were administered into rat tail vein to give a dose of 7.6 $\mu\text{mol/Kg}$ body weight. After 30 min, a blood sample was collected from the heart under anesthesia into heparin-containing tubes, and centrifuged at 4000 g for 8 min at 4°C in order to collect the plasma. Concentrations of the flavonoids in plasma were measured by HPLC with photodiode array detection. Results are expressed as means \pm SEM ($n=5$ animals), with each HPLC measurement performed in duplicate.

^{ab} Different letters show significant differences between flavonoids at $p < 0.05$.

Bars with the same letters indicate no significant difference at $p < 0.05$.

5.2.1.2 Flavonoids distribution in tissues

Figure 5.7 shows the amounts of the different flavonoids detected in liver, kidney, heart, and brain 30 min after administration into the tail vein of rats. The amount of CY per g tissue weight was > 23% higher than the other flavonoids in all evaluated organs. The level of C3G also tended to be higher than QU and Q3G in kidney, although it was not statistically significant ($p=0.16$ and 0.0625 respectively). In some cases such as the levels of the flavonoids in brain and the level of QU in heart, the levels were near the limits of detection.

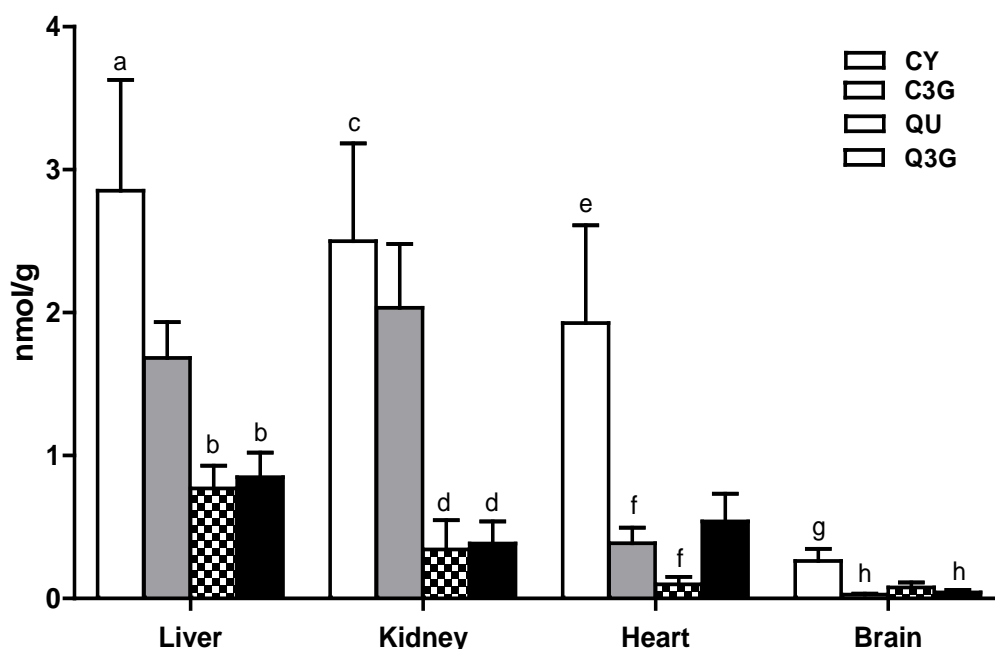


Figure 5.7 Distribution of flavonoids in tissues 30 min after intravenous injection. CY, QU and their 3-glucosides were administered into rat tail vein to give a dose of $7.6 \mu\text{mol/Kg}$ body weight. After 30 min, liver, kidney, heart and brain were removed. Concentrations of flavonoids accumulated in tissues were measured by HPLC with photodiode array detection. Results are expressed as means \pm SEM ($n=5$ animals), with each HPLC measurement performed in duplicate. Representative chromatograms are shown in Appendix B.

ab (liver), cd (kidney), ef (heart), gh (brain)

Different letters show significant differences between flavonoids at $p<0.05$.

Bars with the same letters indicate no significant difference at $p<0.05$.

Comparing the levels in different organs showed some differences. Due to the complexity, the statistical comparisons of the same flavonoid in different organs are described here in the results section rather than in the graph. The recovered CY showed the highest amount in liver with the lowest in brain (liver > kidney > heart > brain), but a statistically significant difference ($p < 0.05$) was found only between liver and brain. C3G accumulated to a higher amount in liver and kidney than heart and brain ($p < 0.05$). QU was distributed more to liver than other organs, and the differences were significant compared to heart and brain contents ($p < 0.05$). Similar results were found with Q3G, but it was significantly different only between liver and brain ($p < 0.05$).

5.2.1.3 Flavonoids distribution into liver mitochondria

After the dose administration, the distribution of flavonoids to the liver mitochondria is shown in Figure 5.8. The concentration of CY recovered in RLM was 10.68 ± 3.38 pmol/mg protein, which was significantly higher than the other flavonoids ($p < 0.05$) (Figure 5.8). No significant difference was found among the other three test flavonoids. Although only a few post-mitochondrial supernatant samples were tested, no flavonoids were detected in post-mitochondrial supernatants.

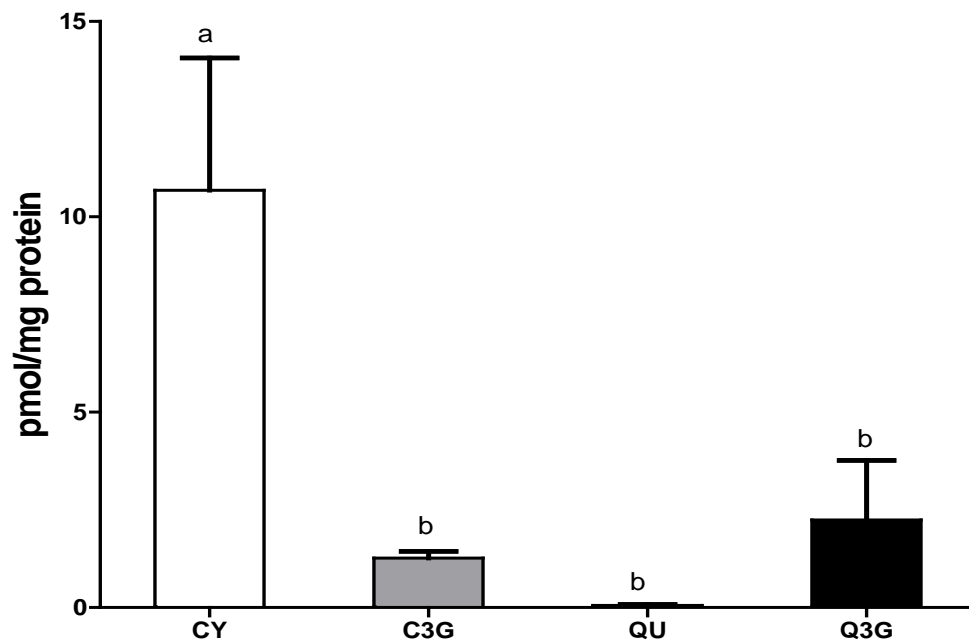


Figure 5.8 Distribution of flavonoids in liver mitochondria. CY, QU and their 3-glucosides were administered intravenously into rat tail vein to give a dose of 7.6 $\mu\text{Mol/Kg}$ body weight. Thirty min after administration, mitochondria were immediately isolated from half of the liver. Concentrations of flavonoids accumulated in mitochondria were measured by HPLC. Results are expressed as means \pm SEM (n=5 animals), with each HPLC measurement performed in duplicate.

^{ab} Different letters show significant differences between flavonoids at $p < 0.05$.

Bars with the same letters indicate no significant difference at $p < 0.05$.

5.2.2 Intravenous flavonoid effects on tissues and mitochondrial oxidative stress

5.2.2.1 Intravenous flavonoid effects on tissue TBARS

The levels of endogenous TBARS in tissues were determined in order to evaluate the effects of intravenous delivery of flavonoids against oxidative stress in the body.

No flavonoid administration groups showed a significant decrease in TBARS (Figure 5.9).

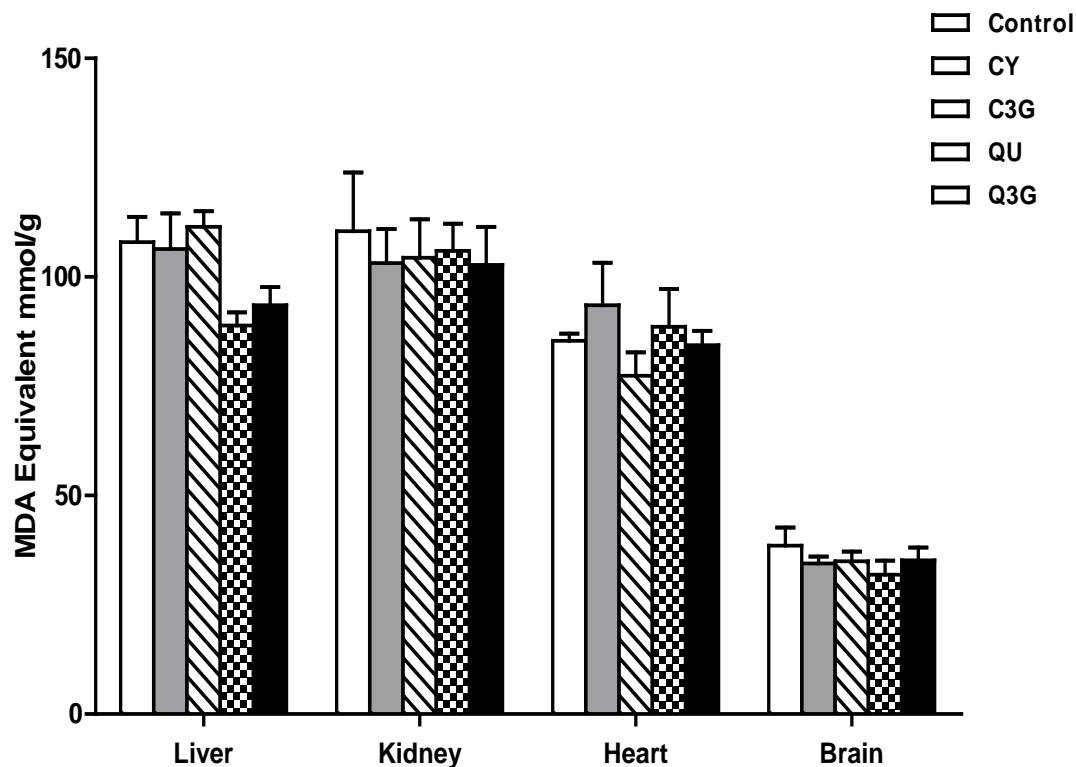


Figure 5.9 Endogenous TBARS in tissues after intravenous flavonoid treatments. CY, QU and their 3-glucosides were administered intravenously into rat tail vein to give a dose of 7.6 $\mu\text{mol/Kg}$ body weight. After 30 min, liver, kidney, heart, and brain were removed. Tissue (about 50 mg) powders were homogenized in 200 μl RIPA buffer (50 mM Tris, 150 mM KCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% EDTA, pH 7.4), and the level of TBARS formation was assayed. Results are expressed as means \pm SEM (n=5 animals), with each TBARS experiment performed in duplicate. In each tissue there were no significant differences from the control group at $p < 0.05$.

In liver, QU and Q3G treatments tended to give a decrease in endogenous TBARS, but it still was not statistically significant ($p=0.2350$ and $p=0.5377$, respectively). The results did indicate that the organs had different degrees of endogenous TBARS, with brain showing the lowest level ($p < 0.05$).

5.2.2.1 Intravenous flavonoid effects on susceptibility of liver mitochondria to lipid peroxidation

The effect of intravenous delivery of flavonoids on susceptibility of the liver mitochondria to lipid peroxidation induced by *t*-buOOH was determined by the TBARS method as described previously (4.3.4). Delivery of the flavonoids at this dose did not significantly affect the susceptibility to lipid peroxidation under the conditions tested (Figure 5.10). There was no decrease in TBARS formation in all flavonoid treatments compared to control group and all experiment groups presented the stimulated peroxidation at an equal level.

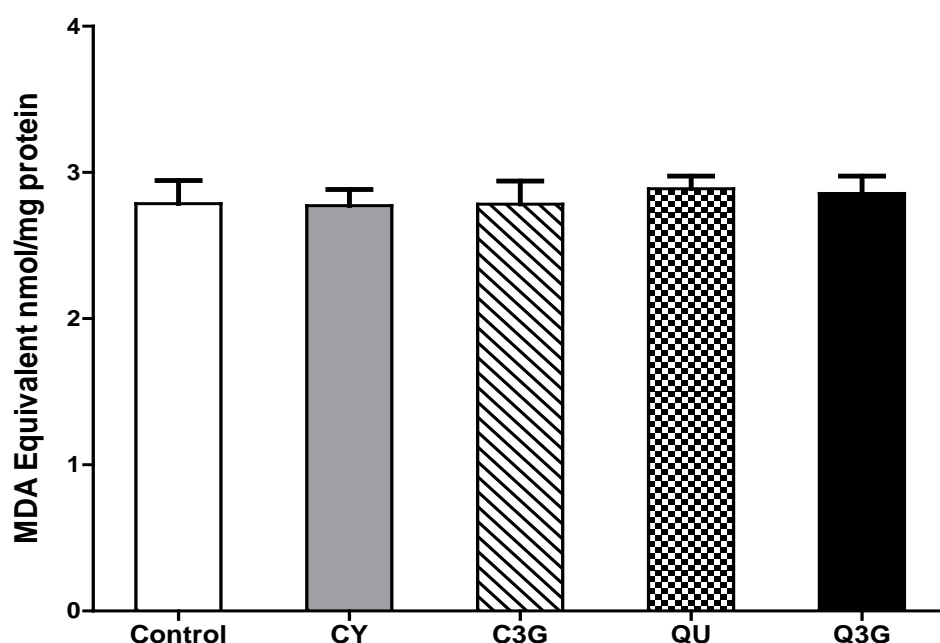


Figure 5.10 Mitochondria susceptibility to lipid peroxidation induced by *t*-buOOH, after intravenous flavonoid treatments. CY, QU and their 3-glucosides were administered into rat tail vein to give a dose of 7.6 $\mu\text{mol/Kg}$ body weight. After 30 min, RLM were immediately isolated from half of the liver. RLM (1 mg/mL of protein) were incubated in 50 mM potassium phosphate buffer (pH 7.2) with 1 mM *t*-buOOH at 37°C for 60 min, and the formation of TBARS was assayed. Results are expressed as means \pm SEM (n=5 animals), with each TBARS experiment performed in duplicate.

There were no significant differences from the control group at $p < 0.05$

6. DISCUSSION

Anthocyanins represent one of the major groups of naturally occurring flavonoids, and are attracting much attention for preventing diseases because of their potent antioxidant abilities and free-radical scavenging activities (de Pascual-Teresa et al., 2010; Heim et al., 2002). A number of studies with anthocyanin-rich foods and extracts have indicated the tissue distribution of anthocyanins and potential beneficial effects in biological systems (Prior & Wu, 2006). However, the biological activities of individual anthocyanins have not been as extensively studied as other flavonoids, in part because of expense and limited commercial sources. In particular, there are no published data reports on the mitochondrial uptake of anthocyanins and their effects on mitochondrial functions. The present thesis investigated cyanidin and cyanidin-3-glucoside uptake in mitochondria and their protective effects against mitochondrial oxidative stress *in vitro*, as compared to the flavonols quercetin and quercetin-3-glucoside. The study also investigated *in vivo* tissue distribution and their accumulation in mitochondria after intravenous administration, and effects on susceptibility of isolated mitochondria to oxidative stress and on endogenous levels of lipid peroxidation products in tissues.

6.1 The ability of flavonoids to accumulate in mitochondria

Flavonoid uptake was investigated in isolated rat liver mitochondria. The uptake of pure cyanidin and quercetin, and their 3-glucosides into isolated rat liver mitochondria were tested with the presence and absence of a respiratory substrate (succinate), as well as with an uncoupler (CCCP) to modulate the membrane potential. The strength of

uptake/binding was also evaluated by BSA incubation prior to centrifugation to remove loosely bound flavonoids. Overall, results indicated that the flavonoid aglycones can rapidly and efficiently enter into or cross mitochondrial membranes in a short time period, especially cyanidin, thereby leading to a marked mitochondria accumulation of the flavonoid (Figure.5.1 and 5.2).

The HPLC results confirmed spectrometric measurements of mitochondrial flavonoid uptake following *in vitro* exposure. In contrast to the HPLC method, the spectrophotometer measurements showed slightly higher levels of uptake. Based on greater accuracy of the HPLC method, these results were used for statistical analysis and discussion. However, the test flavonoids lost during the sample extraction and preparation prior to HPLC injection might be a possible reason that HPLC measurements had lower results.

Previously, anthocyanins have been demonstrated to cross the cell membrane of cultured cells and could be detected in the interior of the cells (McGhie & Walton, 2007), although the mitochondria were not investigated. Evidence also suggests that quercetin can efficiently cross the plasma membrane in many mammalian cell types and is able to reach the mitochondrial compartment (Fiorani et al., 2006; Johnston et al., 2005; Strobel et al., 2005).

Most recently, the rapid accumulation of quercetin in mitochondria has been confirmed (Fiorani et al., 2010). In this study, quercetin accumulated in cells and subsequently accumulated in the mitochondria during incubation of Jurkat human T lymphoblast cells with 10-100 μ M quercetin (Fiorani et al., 2010). The study further showed that when 50 μ M quercetin was incubated for 10 min with purified cellular

mitochondria (0.15 mg protein), mitochondrial quercetin approached the apparent concentration of 35 mM (normalized to mitochondrial volumes) (Fiorani et al., 2010). The present study presented a similar tendency, with 25% of 125 μ M quercetin accumulated in rat liver mitochondria (1 mg protein) after 10 min incubation without BSA. This result provided more evidence of the ability of quercetin to accumulate in mitochondria, employing different test mitochondrial sources.

Flavonoids have an affinity to bind with albumin (Liu et al., 2010). Only a slight non-significant decrease was observed with BSA treatment after incubation with all test flavonoids (Figure.5.3). These results imply strong binding or uptake of flavonoids by the mitochondria, which may involve hydrogen bonds and hydrophobic interactions (Fiorani et al., 2010; Rice-Evans et al., 1996). Flavonoids can accumulate and position at the lipid-water interface of membranes through interactions with, and penetration of cell membranes (Oteiza et al., 2005).

Fiorani et al.(2003) suggested that heme-containing protein allows significant mitochondrial accumulation of flavonoids, this ability of flavonoids to bind with heme-containing protein was also concluded by numerous studies on the effects of phase I monooxygenase enzyme activities (Moon et al., 2006). Mitochondrial hydrophobic membrane and heme-containing protein components may therefore promote preferential accumulation of the flavonoid. Supposedly because of these factors mitochondria express higher uptake and binding ability with quercetin than does cytosol and/or other organelles (Fiorani et al., 2003; Fiorani et al., 2010).

Chemical structure was found to influence flavonoid accumulation in mitochondria in this study. The two aglycones were taken up to a much greater extent than their 3-

glycosylated derivatives under all test conditions (Figure.5.2). Larger molecular size and hydrophilicity of the glucosides make them more difficult to pass through the membrane of mitochondria.

6.2 Anthocyanin accumulation in mitochondria

It was conceivable that anthocyanins and especially anthocyanidins could exhibit appreciable uptake into mitochondria, because they have similar features with TPP cation in being able to bear a positive charge and being lipophilic. Such lipophilic cations accumulate substantially within mitochondria owing to its membrane lipophilicity and potential (Ross et al., 2005). Mitochondrial accumulation of CY was impressive since approximately 67-78% of the initial concentration was recovered in mitochondria after 10 min of incubation. The CY content was confirmed to be significantly higher (3.5 fold) than QU (Figure 5.2.). This finding provided evidence that the anthocyanidin CY rapidly penetrates the mitochondrial membrane and eventually accumulates in mitochondria.

We also found that CCCP significantly decreased the CY content after loading, unlike with the other flavonoids. This evidence suggests that the electrical potential of the mitochondrial membrane is involved in anthocyanidin accumulation to some extent. The large membrane potential (150– 180 mV) across the mitochondrial inner membrane (negative inside) encourages delivery of these molecules to mitochondria, along with the lipophilic character. However, experimental results did not show an increased accumulation of CY when succinate added to the incubation medium. The reason for this is unclear, but the effect of CCCP in the absence of succinate suggests that the mitochondria had a membrane potential due to endogenous substrates (state 1

respiration). In the presence of succinate, CCCP also significantly decreased the CY content, to a level lower than with only CCCP present. The mechanism is not clear, so further investigation is still needed.

Furthermore, the uptake of C3G was significantly lower than CY (5% of 125 μ M), indicating that the hydrophobicity and mass of the molecule are related with accumulation. Addition of CCCP did not have effect on C3G uptake as it did with CY, suggesting that the presence of a glucose moiety may have a stronger influence on anthocyanins accumulation. Apparently, the presence of a sugar residue affects the physicochemical properties of the molecule and thus their ability to cross membranes.

Unlike with CY, the CCCP uncoupling affected only slightly (not statistically significant) the flavonol QU uptake, indicating that the membrane potential may not have much influence this process; thus passive diffusion may be the major route of quercetin accumulation in mitochondria. The difference in effect of CCCP (i.e. decrease in membrane potential) on uptake is likely due to a difference in charge of the flavonoid. At the more acidic pH of the mitochondrial membrane surface, quercetin with a pKa of \sim 7 (Lemanska et al., 2001) would be a mixture of neutral molecules and anions, while cyanidin, with a pKa of 5.5 for the cation (Borkowski et al., 2005), would be a mixture of the neutral quinoid form with the flavylium cation. The chemical property of being more positively charged at physiological pH may be an advantage of anthocyanidins/anthocyanins that leads to uptake being driven to some extent by the mitochondrial membrane potential.

6.3 Effects on mitochondrial respiration

The flavonoids did not have significant effects on mitochondrial oxygen consumption, with succinate or with NADH-producing substrates. These results indicated that the flavonoids did not have strong uncoupling effects, and did not inhibit complex 1 (NADH dehydrogenase) or complex 2 (succinate dehydrogenase) activities. However, the results of Trumbeckaite et al. (2006) suggested that flavonoids possess uncoupling activity in a dose-dependent manner, where the maximal (100%) stimulation of the State 4 respiration was obtained at 1.08 ng/mL (3.6 nM) of quercetin with pyruvate + malate as substrates, but higher concentrations of flavonoids resulted in a drop of uncoupled respiration. In the current study, exposure of mitochondria during state 4 respiration to either 125 μ M CY or QU non-significantly increased oxygen consumption rates 26% and 29%, respectively. Considering the results of Trumbeckaite et al. (2006), the concentration of flavonoids in the current study may have been above that where significant uncoupling would be observed. More recently, Trumbeckaite et al. (2012) reported that the anthocyanins, malvidin-3-glucoside, malvidin-3-galactoside, and cyanidin-3-galactoside at ~50 nM had no effects on rat heart mitochondrial respiration with pyruvate plus malate.

6.4 The effects of flavonoids on mitochondrial oxidative damage

6.4.1 Antioxidant ability of flavonoids incubated together with mitochondria

The impacts of flavonoids on mitochondrial oxidative stress were evaluated by ROS generation and TBARS formation measurements.

The effects of the different flavonoids were compared at equimolar concentration on antimycin-stimulated ROS generation during mitochondrial respiration. Consistent with

their known effects on mitochondrial ROS generation (Cadenas and Davies 2000) antimycin stimulated ROS generation by 39%, while CCCP decreased ROS generation by 34%. Cyanidin, quercetin and their 3-glucosides all strongly inhibited antimycin stimulated ROS generation (Figure 5.4). The flavonoids all inhibited ROS generation and/or accumulation to levels well below that in the presence of CCCP. The flavonoids may have either modulated ROS generation or effectively scavenged ROS, or worked both ways. Additionally, when added at equimolar concentrations QU showed a significantly stronger effect on ROS generation than CY, which may have been due to QU being a very efficient radical scavenger (Rice-Evans et al., 1996) or due to an effect on the generation of ROS by the respiratory chain. However, in the preloading experiments, CY gained a greater effect on ROS generation due to greater accumulation.

When added externally to RLM, CY showed a significant lowering effect on ROS generation when compared to C3G. This finding leads to the hypothesis that although less C3G than CY is taken up by mitochondria, C3G might be an efficient antioxidant in cells against oxidative damage caused by mitochondrial release of ROS.

Similar effects of flavonoids on ROS generation in the presence of antimycin were also reported most recently (Lagoa et al., 2011). With much lower concentrations of flavonoids than were used in this study, although also with a much lower concentration of mitochondria (0.1 mg protein/mL), quercetin effectively inhibited ($IC_{50} \approx 1-2 \mu M$) H_2O_2 production stimulated by rotenone and antimycin in rat heart and brain mitochondria. Also, the study found that 10 μM quercetin decreased the state 3 (absence of antimycin) H_2O_2 production rate in rat heart mitochondria (0.1 mg/mL

protein) to 30% and in brain mitochondria (0.1 mg/mL protein) to <10%. However this study failed in showing the effect of cyanidin because of direct interference with the Amplex Red/peroxidase detection method.

The four flavonoids were also compared for their ability to inhibit *t*-buOOH-induced mitochondrial lipid peroxidation. All test flavonoids significantly suppressed the *t*-buOOH induced lipid peroxidation to a similar extent (Figure 5.4), confirming that these flavonoids and their 3-glucosides were powerful chain-breaking antioxidants that protect membranes from oxidative damage (Oteiza et al., 2005).

In addition, besides free radical scavenging ability, the hydroxyl moieties of the B and C rings allow good metal chelation ability (Welch et al., 2008). Low molecular weight Fe^{2+} complexes exist in mitochondria and are capable of catalyzing mitochondrial membrane lipid peroxidation (Valko et al., 2006).

Flavonoids have previously shown protective effects against mitochondrial lipid peroxidation. Experiments on isolated rat liver mitochondria indicated that quercetin had strong potency ($\text{IC}_{50}=30\ \mu\text{M}$) in inhibiting ADP/Fe(II)-induced lipid peroxidation (Santos et al., 1998). Our results support previous observations that flavonols, anthocyanins and anthocyanin aglycones are effective at inhibiting transition metal-catalyzed lipid membrane peroxidation (Soczynska-Kordala et al., 2001).

Anthocyanins have previously shown protection against peroxidation in other lipid systems. Wang et al. (1999) reported that cyanidin and three cyanidin glycosides inhibited lipid peroxidation of a liposome system by 39% to 75%, consistent with results in our study. Tsuda et al., (1994) reported that cyanidin had a stronger effect than C3G in preventing lipid peroxidation induced by active oxygen radicals in

liposomes (linoleic acid autoxidation) and rabbit erythrocyte membranes. According to these results, glycosylation with carbohydrate units at the C3 position decreases antioxidant ability. However this observation was not confirmed in the current study.

There still is limited information on antioxidant activities and free radical scavenging properties of anthocyanins compared to studies of other flavonoids. Overall, this study provided evidence that anthocyanins might have similar protection effects as flavonols against oxidative lipid damage, although at the concentration employed (125 μ M) the *t*-buOOH-induced TBARS formation was completely blocked by all flavonoids.

6.4.2 Bio-antioxidant ability of flavonoids after accumulation in mitochondria

When mitochondria were incubated together with added flavonoids (discussed above), the impact of evaluated flavonoids on induced mitochondrial ROS and oxidative damage was suppressed to a similar extent. But the whole protective effect was partly contributed by flavonoids which were not taken up in mitochondria and present in the medium. Our results demonstrate that some flavonoids can accumulate in mitochondria to a remarkable amount in a short time. It is important to know the extent that the mitochondria preloaded flavonoids could display biological activity and protect mitochondria from oxidative damage. The test on flavonoid- preloaded mitochondria may be a more relevant expression to the antioxidant ability of the flavonoids in the biological system.

Flavonoids preloaded into mitochondria were successful in protecting against induced ROS and oxidative damage. Accumulated flavonoids in mitochondria effectively modulated/interfered with induced ROS generation and lipid peroxidation

(Figures 5.4 and 5.5); with CY and QU showing the strongest activities. The preloaded flavonoid glucosides, especially C3G, gave less protection than the aglycones, which was consistent with the observation that the glucosides had less accumulation in mitochondria. Part of this difference may be that substitution of hydroxyl groups by glycosylation decreases the antioxidant activity (Havsteen, 2002; Rice-Evans et al., 1996; Wang et al., 1999), and may influence the antioxidant capacity of flavonoids in lipid oxidation systems. However glycosylation also influences the lipophilicity/hydrophilicity and decreased their mitochondrial uptake, which would decrease their effect on membrane lipid peroxidation.

The measurement of ROS generation by mitochondria with flavonoids preloaded showed CY to be the most protective, although with similar protective effects by QU. While the stronger protection by CY reflects its higher concentration (i.e. uptake) in the mitochondria and its strong free radical scavenging properties, the almost equal protection by QU despite less being present suggests that QU is more potent at lowering ROS levels. Similar TBARS formation suppression was also observed in CY and QU preloaded mitochondria, supporting the greater potency of QU against oxidative damage.

Preloaded flavonoid glucosides, which showed a lower level of mitochondrial accumulation, seemed to moderate ROS generation to a similar level as their preloaded aglycones. Considering the lower uptake, the flavonoid glycosides may display stronger biological activities against ROS generation. However, their mechanism of this impact is not clear, possible actions are effects on the mitochondrial electron transport chain and scavenging generated ROS. Further investigation is still needed.

Overall, the *in vitro* study demonstrated that cyanidin can rapidly accumulate in mitochondria which was postulated to be due in part to its unique chemical structure. The test flavonoids accumulated in mitochondria exhibited significant antioxidant ability in the presence of oxidative stress. Mitochondria are a critical source of ROS generation, so their ability to accumulate a significant amount of cyanidin suggests that cyanidin could be potential mitochondria protective compound against oxidative damage in physiological systems.

6.5 Flavonoids distribution in tissues, mitochondria and plasma *in vivo*

Several studies employing fruit extracts have demonstrated that anthocyanins are rapidly absorbed and distributed in the blood and tissues (Table 2.2). So far, some studies suggest the core phenolic aglycones and conjugated sugar moieties can influence the absorption of anthocyanins (Ichiyanagi et al., 2006; Kalt et al., 2008; Wu et al., 2005), but no investigations have been performed comparing anthocyanin glycones and aglycones as well as comparing anthocyanins to other flavonoids.

Our results provide evidence that the pure anthocyanidin and its glucoside have a greater ability to accumulate in organs, especially liver and kidney, than the analogous flavonols (Figure 5.5). In liver, the content of CY was significantly higher (2.85 nmol/g tissue weight) than QU (0.77 nmol/g) and Q3G (0.85 nmol/g) ($p < 0.05$). Similarly, anthocyanin distribution in the kidney with C3G of 2.03 nmol/g and CY of 2.50 nmol/g, was higher than that of QU and Q3U (< 0.38 nmol/g). These results were higher than the results of Ichiyanagi et al. (2006), who reported total anthocyanins of 0.14 nmol/g and 0.46 nmol/g in liver and kidney tissue, respectively, 15 min after intravenous administration of a 5 mg bilberry extract (equal to a dose of 11.1 $\mu\text{mol/Kg}$

body weight anthocyanins, which was slightly higher than our dose of 7.6 $\mu\text{mol/Kg}$ body weight. The used anthocyanins source and administration time were possibilities for the differences in tissues distribution

The CY and C3G levels in plasma of 0.61 and 0.68 $\mu\text{mol/L}$ were lower than those of QU and Q3G (1.88 and 1.69 $\mu\text{M/L}$) 30 min after intravenous delivery, suggesting more rapid clearance and/or tissue uptake of the anthocyanins. These results with the anthocyanins were slightly higher than but similar to those of Matsumoto et al. (2006a) who reported a C_{max} of 0.285 $\mu\text{mol/L}$ and T_{max} of 26.3 min in plasma after the same intravenous dose of delphinidin-3-rutinoside from blackcurrant extract.

Quantitative comparisons of tissue distribution also indicated the higher amount of CY than C3G with the same dose administration. Glycosylation influences both the molecular size and lipophilicity of anthocyanins; and thus influences their ability to be taken up by, and enter into tissue cells.

Compared to brain, CY and C3G accumulated to a significantly higher amount in liver tissue. These findings among tissues are consistent with anthocyanin and anthocyanidin absorption, metabolism and retention among tissues and factors that delay distribution such as the blood-brain barrier and the blood-retinal barrier (Kalt et al., 2008). A similar observation was also found for QU and Q3G, which QU distributed more in liver than other organs, and significant differences were found compared to heart and brain contents. Clearly, these results show that the flavonoid accumulation is tissue dependent.

As was noted, CY and to a lesser extent C3G is more effectively accumulated in tissues than the flavonols. In addition, CY accumulated more in mitochondria than the

flavonols, consistent with the *in vitro* results. These findings demonstrated that cyanidin was associated with tissue cells and accumulated even further in mitochondria *in vivo* because of its unique chemical structure properties. The relatively lower concentration of QU in tissues and mitochondria and higher plasma concentration also indicates cyanidin is more rapidly taken up into mitochondria.

However, there are some concerns in the present study. Firstly, as discussed in 5.2.3, the recovery rate of the tissue extraction recovery method is not satisfying. The anthocyanins recovery from liver was $57 \pm 19\%$, and from kidney was $89 \pm 13\%$ in Kalt et al.'s study (Kalt et al., 2008), which used a modified extraction method from the Tsuda group (Tsuda et al., 1999b). Whereas, with different extraction procedures and solvents used, our flavonoid tissue recovery was only about 23%-40%, the concentration in tissues might be substantially underestimated. Also, Kalt et al. (2008) reported that freeze drying and storage $-80\text{ }^{\circ}\text{C}$ causes anthocyanin losses. Therefore the sample extraction methods may not have been ideal to detect such low content of flavonoids. In addition, the flavonoid distribution *in vivo* was low, and the test sample concentration of some flavonoids (QU and Q3G) were actually close to the HPLC limit of quantification range (Appendix B), especially in the measurements from mitochondria. Thus, in order to confirm these results, future studies with a better sample preparation method are required. Nevertheless the levels of CY in tissues and mitochondria were high enough to be readily measured, and showed the relatively higher accumulation compared to the other flavonoids.

6.6 Considerations of results analysis in the *in vivo* study

Although flavonoid recovery from RLM (both *in vivo* and *in vitro*) were >86%, the extraction recovery of the flavonoids for tissues were <40% (Appendix Table B.1), so the tissue concentrations might be substantially underestimated. Also, the HPLC column was regenerated and not very efficient. Therefore, the responding spectral peak area of some of the flavonoids were close to or lower than the HPLC limit of quantification range, especially during mitochondria measurements after *in vivo* treatments (except for CY). No correction was made in the results for losses during sample extraction. To confirm these results, future studies with a better sample preparation method are required.

6.7 Flavonoid effects on tissues and mitochondrial oxidative stress

We also tested the extent to which the intravenous delivery of flavonoids protected the tissues and mitochondria from oxidative damage. A few studies have documented anthocyanins administration effects on cell and tissues oxidative stress. The oral administration of an anthocyanin-rich extract prevented hypertension, cardiac hypertrophy and production of ROS, and expression of NADPH oxidase in rats maintained on a high-fructose diet (Al-Awwadi et al., 2005). In a study on *in vivo* antioxidant activity of C3G by using hepatic ischemia/ reperfusion (I/R) injury as a model of oxidative stress, the C3G oral administration significantly suppressed rat liver damage caused by increased oxidative stress (Tsuda et al., 1999a). In this perspective, anthocyanins administration, which our results show subsequently accumulate in tissues and further in mitochondria, can sufficiently increase antioxidant capacity of tissues against oxidative damage.

In the current study with intravenous injection of flavonoids to rats, it indicated the lack of significant protection effect of flavonoid administration on tissue endogenous oxidative stress. This is probably due to low flavonoid content achieved in this protocol. Also, the results failed to support the initial hypothesis, there were no effects on endogenous TBARS after 30 min administration. Another approach might be to test for susceptibility to TBARS formation in order to evaluate the flavonoid effects on tissues oxidative stress. Susceptibility to TBARS formation could not be tested in the current homogenates in RIPA buffer due to the presence of EDTA, which would inhibit metal-catalyzed lipid peroxidation.

Moreover, our results indicated that organs had different degrees of endogenous lipid peroxidation, with brain showing the lowest level. The mechanism of such tissue dependent TBARS levels is not clear yet.

With mitochondria, intravenous delivery of flavonoids did not affect mitochondrial susceptibility to lipid peroxidation (Figure 5.8). Considering the low amount of flavonoids accumulated in mitochondria after delivery, it might not have sufficient effect against oxidative stress to be observed in experiments with this single dose administered intravenously. Another possibility for this unexpected result may be the quite high *t*-buOOH used, which may have overwhelmed the loaded flavonoids. The test methods need to be modified in order to give better future determinations.

7. CONCLUSIONS AND PERSPECTIVE

Anthocyanins such as cyanidin are an important class of flavonoid and abundant in the diet. The present study provides evidence that cyanidin acts as a mitochondrial targeted antioxidant and, thereby, exhibits protective effects. Cyanidin can rapidly and efficiently cross mitochondrial membranes in a short time period, leading to a remarkable mitochondria accumulation. With regards to the antioxidative mechanism of anthocyanins, the results suggest that while anthocyanidins such as cyanidin accumulate in mitochondria, they exhibit strong bio-antioxidant activity against oxidative stress. Also, the presence of sugar residues affects the physico-chemical properties of the molecule and thus their ability to cross membranes and enter mitochondria. According to these results, cyanidin could be a potent natural antioxidant compound that is effective in mitochondria-protective therapies.

The *in vivo* study indicates that the anthocyanidin and its glucoside have greater ability than flavonols to accumulate in organs; especially liver and kidney. Also, the results indicated cyanidin distributed more in mitochondria than the other flavonoids and consistent with *in vitro* results was present in mitochondria to a much greater extent than its glycoside.

Perhaps because of low concentration, a single intravenous cyanidin administration resulted in no statistically significant protection on tissue oxidative stress. Also with mitochondria, no significant effects of flavonoids delivery were found in susceptibility to lipid peroxidation induced by *t*-buOOH due to the low concentration.

In conclusion, with emphasis to the mitochondrial antioxidant activity of anthocyanins, cyanidins as dietary compounds have potential beneficial effects on human health and can apply in numerous disease conditions. The mitochondrial targeted protective properties of cyanidin suggest that anthocyanidins are of possible therapeutic use in mitochondria diseases.

8. FUTURE DIRECTIONS

My thesis indicated the ability of CY to accumulate in mitochondria; more studies are still needed to determine the mechanisms of this action. CY showed a significant slightly lowering effect on ROS generation than C3G and QU, but with similar effects as the other flavonoids against lipid peroxidation. Further investigation might need to establish the individual anthocyanins antioxidant properties in different types of oxidative damage. Structure-activity relationships need further investigations.

Further studies are needed to complete the quantification of flavonoids in tissues, cells and mitochondria using more suitable analysis methods. A more specific method to extract flavonoids from tissue samples is needed.

In the current *in vivo* study, we learned about flavonoid distribution in plasma, tissues and mitochondria with single dose of administration. It will be important to measure with higher doses and different time periods after administration. Also, the tissue and mitochondria distribution investigation would need to be extended to different delivery methods including oral, gavage and dietary. Data on long term administration and different doses of delivery are needed.

The protective effects of flavonoids in tissues and mitochondrial oxidative damage were not observed in this study. The low dose administration with consequently low distribution into mitochondria is one possible explanation. A higher or multiple dose protocol might lead to greater flavonoids accumulation and exhibit expected effects. Alternatively, a more gentle stress should be employed, such as incubation in air (instead of with 5 mM *t*-buOOH) might be needed to reveal protective effects.

To further reveal the flavonoids effects on oxidative stress, a long term dietary administration investigation would be important and with different doses and time period of treatment as well as other delivery protocols.

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Appendix A. Mitochondrial uptake of flavonoids spectrometric method

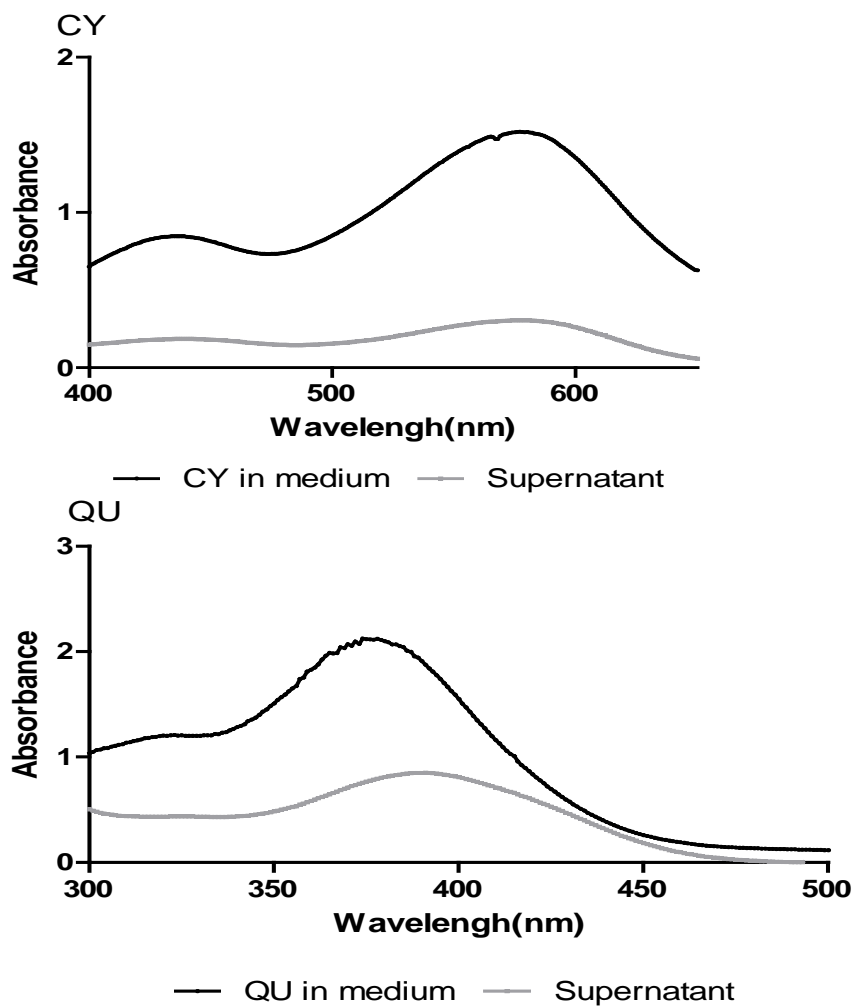


Figure A.1 Mitochondrial uptake of cyanidin and quercetin. Example tests of spectrophotometric measurements. Scan of cyanidin or quercetin (125 μ M) in medium before and after incubating with RLM and centrifuging.

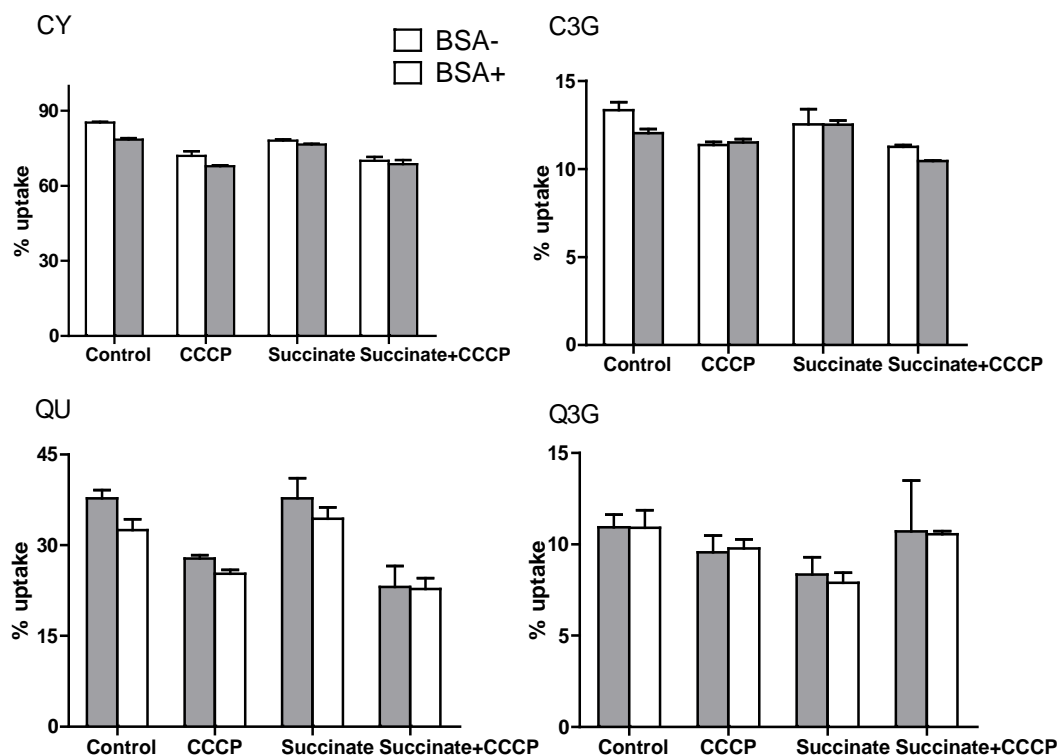
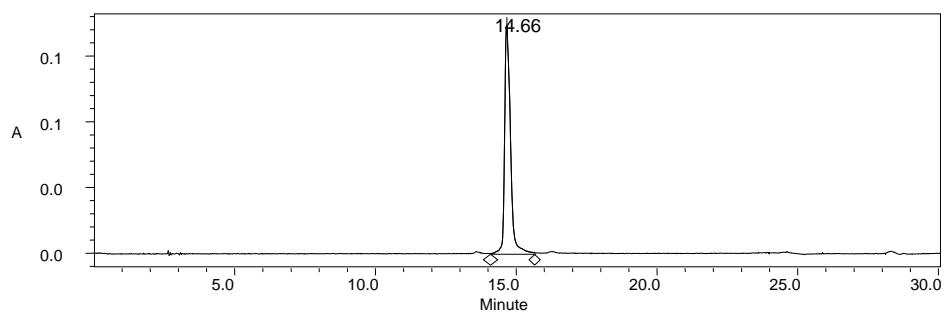


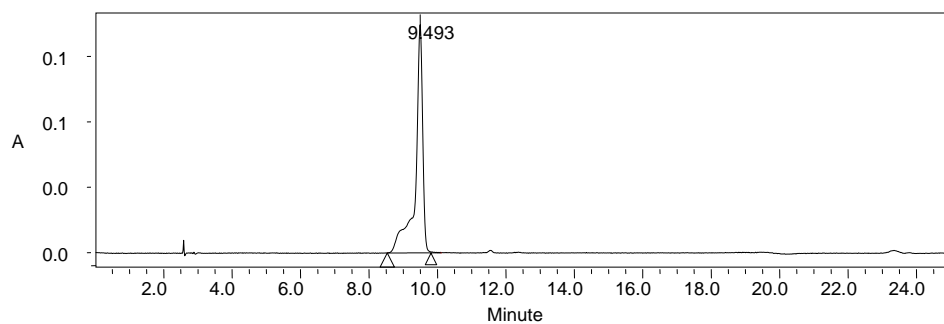
Figure A.2 Resistance of mitochondrial bound/uptaken flavonoids to removal by BSA. Percent (%) uptake of flavonoids (125 μ M) by RLM (1 mg protein) were measured by the spectrophotometric method. Experiments were conducted in 1 mL sucrose-HEPES buffer in the presence and absence of a respiratory substrate (succinate), and an uncoupler (CCCP) and were compared between with BSA and without BSA added before centrifugation. Results are expressed as means \pm SEM (n=3 mitochondrial preparations), with each experiment performed in triplicate. There are no significant differences between with BSA and without BSA treatment in measurements of any flavonoids.

Appendix B. HPLC analysis chromatograms and recoveries

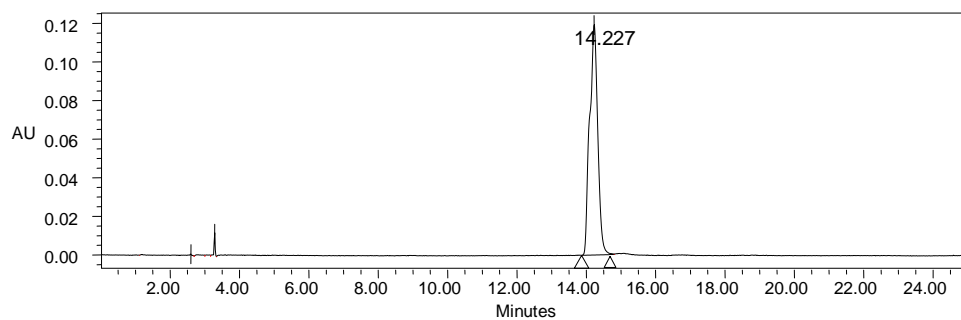
CY



C3G



QU



Q3G

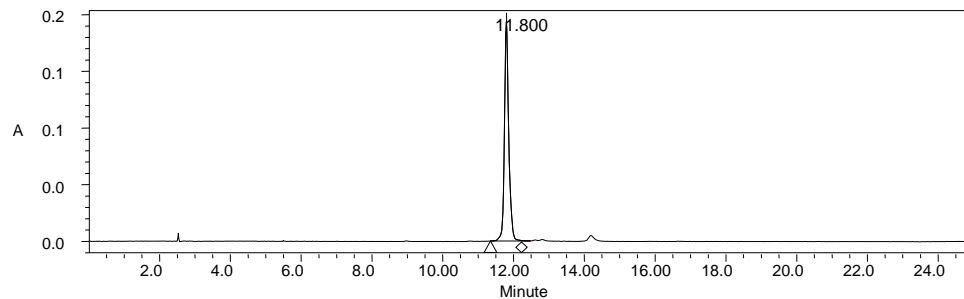
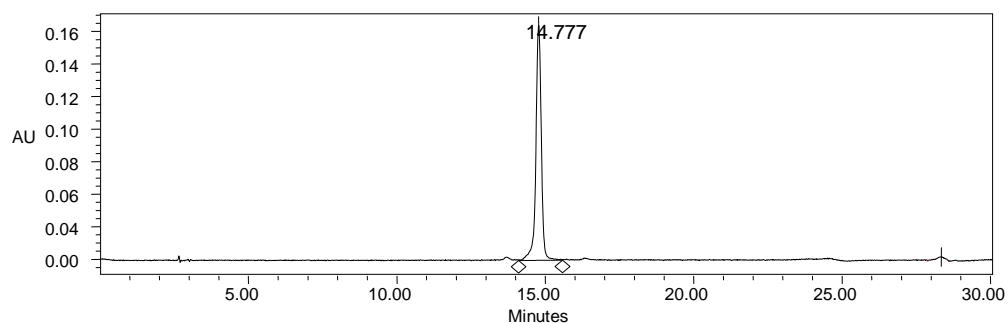
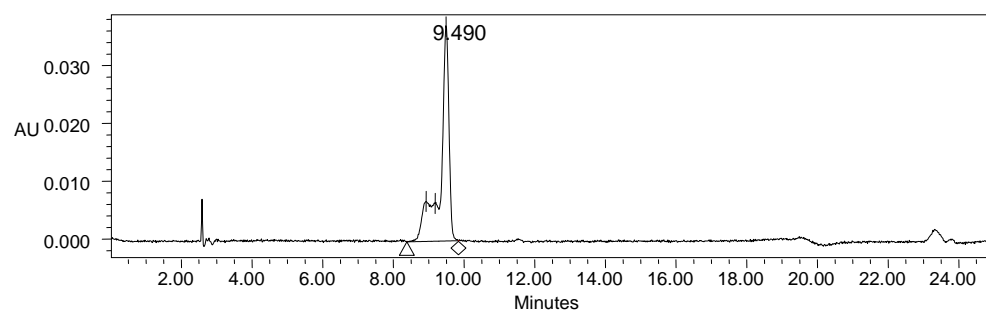


Figure B.1. Chromatogram of flavonoids standard solution (60 μ M)

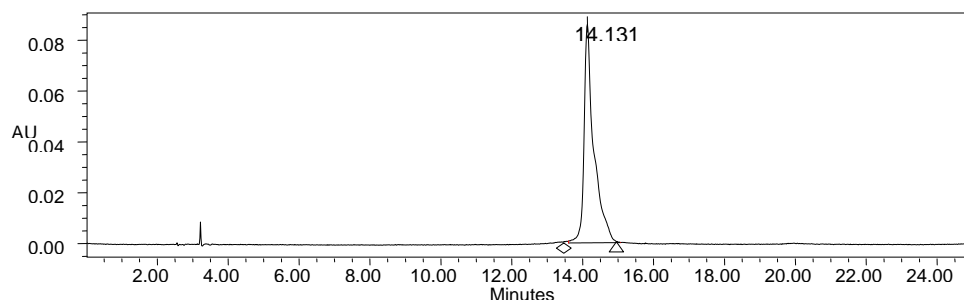
CY



C3G



QU



Q3G

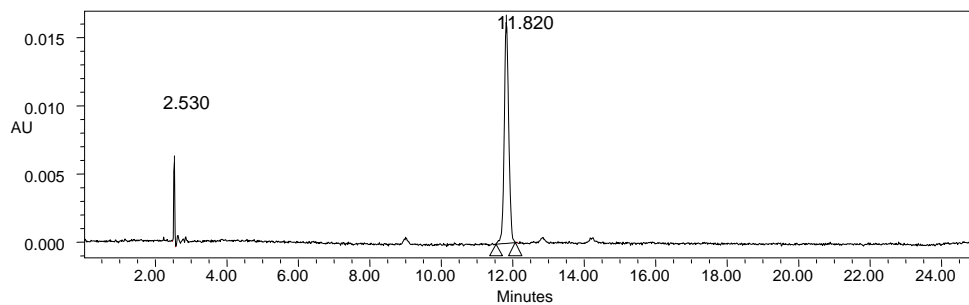
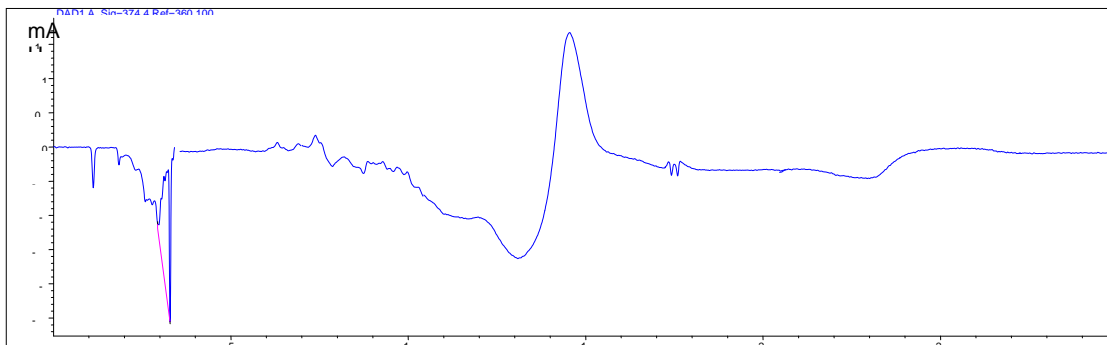


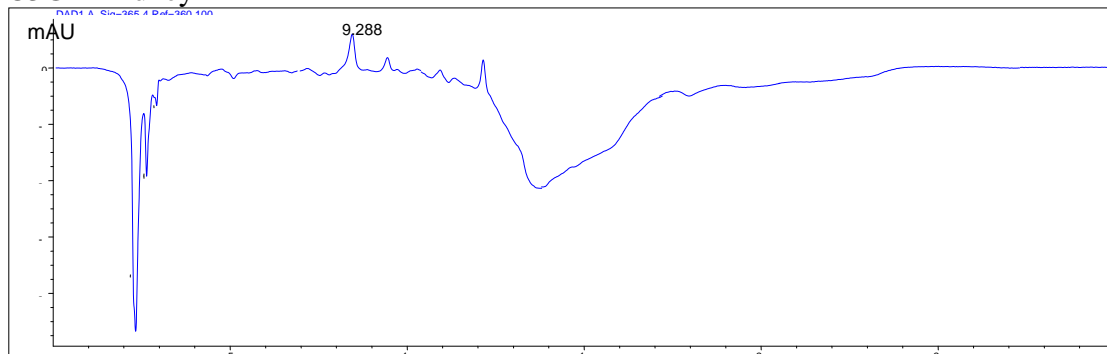
Figure B.2 HPLC chromatogram of mitochondrial uptake (with BSA).

Examples chromatograms of extractions from RLM pellet after flavonoids incubation.

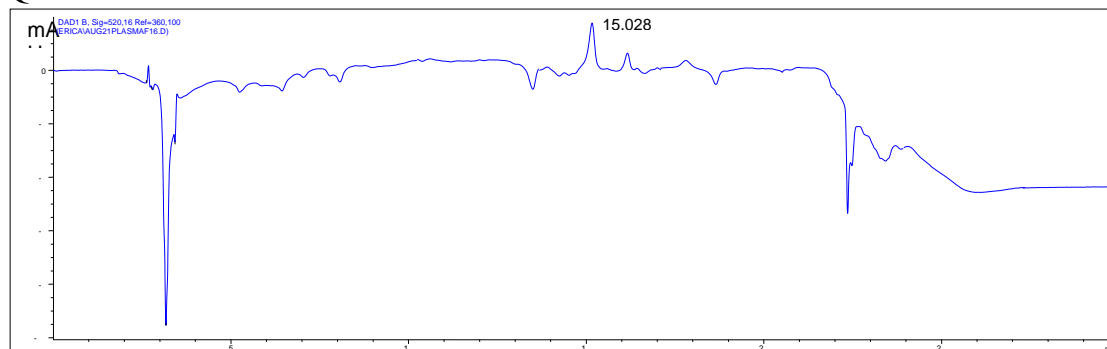
CY in liver



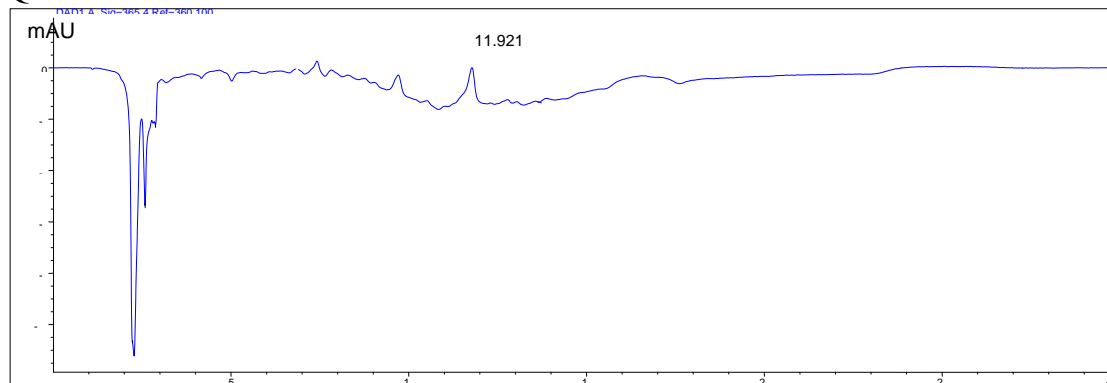
C3G in kidney



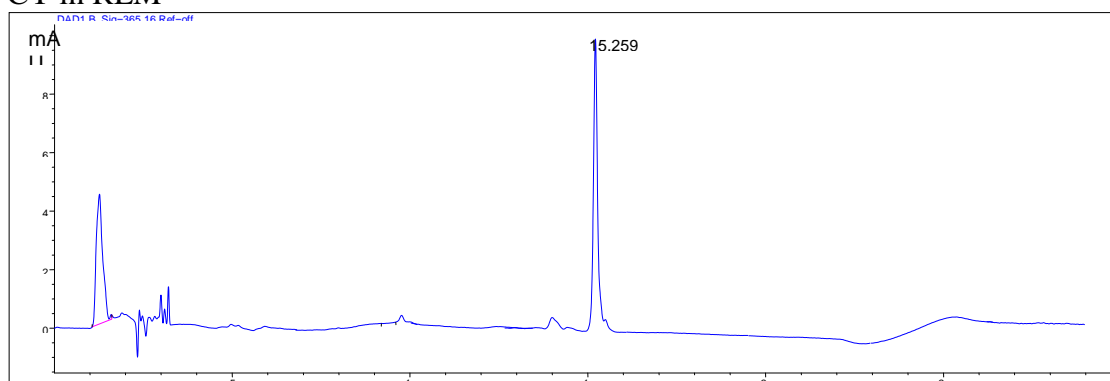
QU in liver



Q3G in heart



CY in RLM



QU in plasma

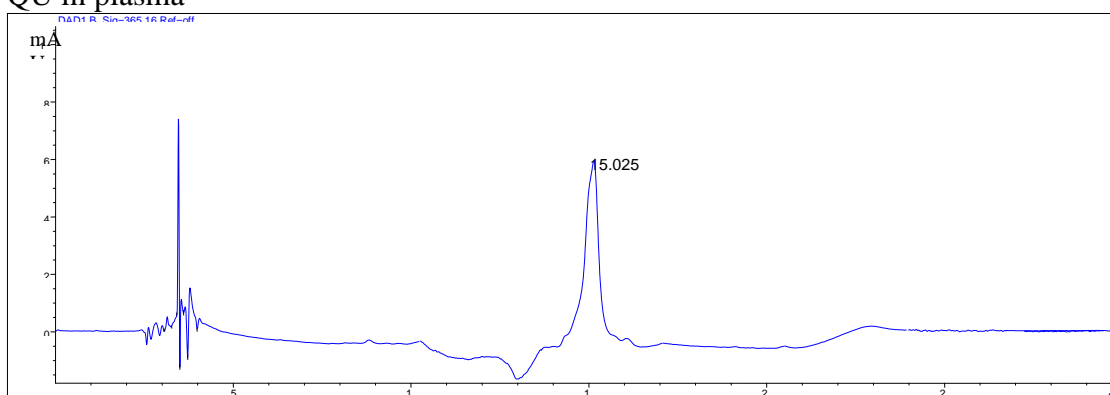


Figure B.3 HPLC chromatogram of flavonoid in tissues, RLM and plasma.

Example chromatograms of extractions from tissues, RLM and plasma after intravenous administration into rat tail vein to give a dose of $7.6 \mu\text{mol/Kg}$ body weight flavonoids.

Table B.1 HPLC analysis parameters and recovery of test flavonoids in tissues, plasma and mitochondria. Results are expressed as means \pm SEM (n=3 preparations), with each experiment performed in triplicate.

Recovery %							
Compound	Liver	Kidney	Brain	Heart	Plasmas	RLM (<i>in vivo</i> study)	RLM (<i>in vitro</i> study)
CY	33.41	32.88	35.92	25.47	79.33	87.43	92.48
C3G	26.07	30.36	33.85	22.84	81.28	91.12	86.35
QU	32.89	34.98	39.63	32.77	80.73	87.94	90.72
Q3G	28.89	29.02	31.86	30.23	85.27	92.87	93.55
Compound	Limit of detection (μ M)			Limit of quantification (μ M)			
CY	0.3			1.5			
C3G	0.3			1.5			
QU	0.2			1			
Q3G	0.2			1			

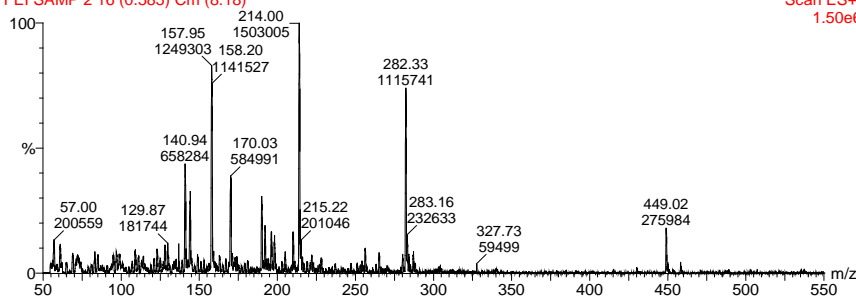
Appendix C. Mass spectrometry analysis of C3G

15 μ M

Capillary 3.5 Cone 30, Gain 10, Source Temp 140, Fei Sample 1

FEI SAMP 2 16 (0.585) Cm (8:18)

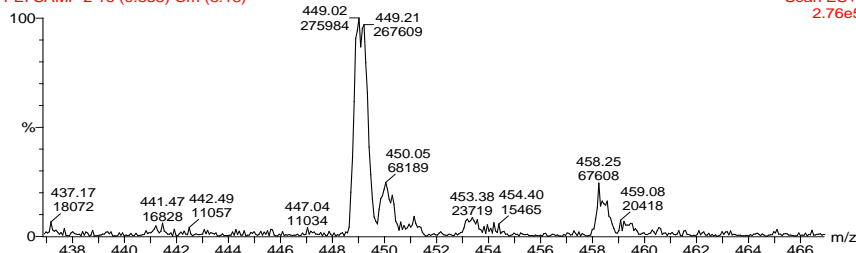
Scan ES+
1.50e6



Capillary 3.5 Cone 30, Gain 10, Source Temp 140, Fei Sample 1

FEI SAMP 2 16 (0.585) Cm (8:18)

Scan ES+
2.76e5

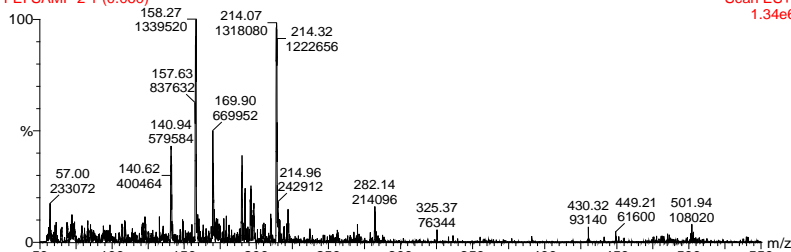


1.5 μ M

Capillary 3.5 Cone 30, Gain 10, Source Temp 140, Fei Sample 1

FEI SAMP 2 1 (0.060)

Scan ES+
1.34e6



Capillary 3.5 Cone 30, Gain 10, Source Temp 140, Fei Sample 1

FEI SAMP 2 1 (0.060)

Scan ES+
2.14e5

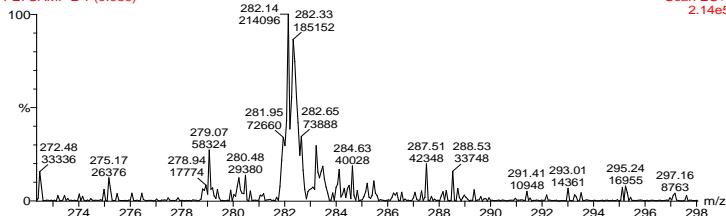


Figure C. Chromatogram of mass spectrometry analysis of C3G. During the HPLC identification and quantification analysis, we found a shoulder present in the chromatogram of C3G. We performed a mass spectrometry analysis on C3G, and confirmed it was caused by the presence of isomers. Thus, it would not influence the investigation.